

PATENT COOPERATION TREATY

REC'D 22 OCT 2002

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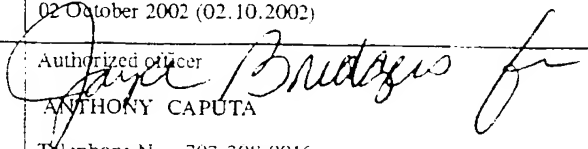
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

JAN 03 2003

(PCT Article 36 and Rule 70)

TECH CENTER 1600/29

Applicant's or agent's file reference WST97APCT		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/16391	International filing date (day/month/year) 14 June 2000 (14.06.2000)	Priority date (day/month/year) 29 July 1999 (29.07.1999)	
International Patent Classification (IPC) or national classification and IPC IPC(7): A01N 63/00; A61K 35/18; C12N 5/02, 5/08; C07K 2/00 and US Cl.: 424/93.73,533; 435/372,384,397,389,405.			
Applicant THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of <u>5</u> sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of <u>2</u> sheets.</p> <p>3. This report contains indications relating to the following items:</p> <p>I <input checked="" type="checkbox"/> Basis of the report</p> <p>II <input type="checkbox"/> Priority</p> <p>III <input type="checkbox"/> Non-establishment of report with regard to novelty, inventive step and industrial applicability</p> <p>IV <input checked="" type="checkbox"/> Lack of unity of invention</p> <p>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p>VI <input type="checkbox"/> Certain documents cited</p> <p>VII <input type="checkbox"/> Certain defects in the international application</p> <p>VIII <input type="checkbox"/> Certain observations on the international application</p>			
Date of submission of the demand 07 February 2001 (07.02.2001)		Date of completion of this report 02 October 2002 (02.10.2002)	
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230		Authorized officer  ANTHONY CAPUTA Telephone No. 703-308-0916	

Form PCT/IPEA/409 (cover sheet)(July 1998)

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/16391

I. Basis of the report

1. With regard to the elements of the international application:*

- ☒ the international application as originally filed.
- ☒ the description:
pages 1-47 as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the claims:
pages 48-55, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the drawings:
pages 1-12, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the sequence listing part of the description:
pages 1-17, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

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2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in printed form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

- ☐ the description, pages NONE
- ☐ the claims, Nos. NONE
- ☐ the drawings, sheets/fig NONE

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

** An replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/16391

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The question whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

- ☐ the entire international application,
☒ claims Nos. 14-20, 26, 28, 29, 31, 33-37 and 39-42

because:

- ☐ the said international application, or the said claim Nos. _____ relate to the following subject matter which does not require international preliminary examination (*specify*):

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- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. _____ are so unclear that no meaningful opinion could be formed (*specify*):

- ☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.
☒ no international search report has been established for said claims Nos. 14-20, 26, 28, 29, 31, 33-37 and 39-42

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
☐ the computer readable form has not been furnished or does not comply with the standard

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/16391

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
☐ paid additional fees.
☐ paid additional fees under protest.
☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied with and chose, according Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention is accordance with Rules 13.1, 13.2 and 13.3

- ☐ complied with.
☒ not complied with for the following reasons:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-6, 11-13, 21-23, drawn to an isolated nucleic acid sequence encoding a chfr protein, or an anti-sense thereof a method of determining tumorigenic potential of a cell comprising examining the presence of chfr nucleic acid sequence.

Group II, claim(s) 7-10, drawn to a chfr protein.

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4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report.

- ☐ all parts.
☒ the parts relating to claims Nos. 1-13, 31-25, 27, 30, 32, 38

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/16391

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability, citations and explanations supporting such statement

1. STATEMENT

Novelty (N)

Claims 2-13, 21-25, 27, 30, 32 and 38

Claims 1

Inventive Step (IS)

Claims 2-13, 21-25, 27, 30, 32, 38

Claims 1

Industrial Applicability (IA)

Claims 1-13, 2-25, 27, 30, 32, 38

Claims NONE

2. CITATIONS AND EXPLANATIONS

Claim 1 lacks novelty under PCT Article 33(2) as being anticipated by Scolnick et al.

Scolnick et al teach a chfr gene that is involved in the spindle checkpoint, that delays the condensing of the chromosome. Thus the gene taught by the art is the same as the claimed chfr gene.

Claims 2-13, 21-25, 27, 30, 32, 38 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a polypeptide of SEQ ID NO:2. Further, since the sequence structure of the chfr gene is not taught in the art, the method use of said gene and the encoded polypeptide are not obvious.

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PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
 US Department of Commerce
 United States Patent and Trademark
 Office, PCT
 2011 South Clark Place Room
 CP2/5C24
 Arlington, VA 22202
 ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 09 November 2001 (09.11.01)	
International application No. PCT/US00/16391	Applicant's or agent's file reference WST97APCT
International filing date (day/month/year) 14 June 2000 (14.06.00)	Priority date (day/month/year) 29 July 1999 (29.07.99)
Applicant HALAZONETIS, Thanos et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

07 February 2001 (07.02.01)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date on, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Antonia MULLER Telephone No.: (41-22) 338.83.38
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/16391

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/04; C07K 16/00

US CL :536/23.1; 530/387.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MPSRCH, DIALOG, WEST

search terms: chfr, cancer, forkhead-associated protein, Ring finger

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	SCOLNICK et al. CHFR prevents chromosomal condensation in response to a defective spindle. Proceedings Amer. Assoc. Cancer Res. March 1999, Vol. 40, page 214, abstract No. 1422. See entire document.	1,7 ----- 3,4,11-13
Y	MURONE et al. The fission yeast dma1 gene is a component of the spindle assembly checkpoint, required to prevent septum formation and premature exit from mitosis if spindle function is compromised. EMBO J. 02 December 1996, Vol. 15, No. 23, pages 6605-6616. See entire document.	23-25
X,P	Database GenBank, Accession No. AK001658, ISOGAI et al. 'NEDO human cDNA sequencing project'. Publicly available 16 February 2000. See entire document.	23-25

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"G" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 12 FEBRUARY 2001	Date of mailing of the international search report 27 APR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Jane Bridges</i> MINH TAM DAVIS Telephone No. (703) 308-0916

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/16391

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-13,21-25,27-30,32,38
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.

Remark on Protest



The additional search fees were accompanied by the applicant's protest



No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-6, 11-13, 21-22, drawn to an isolated nucleic acid sequence encoding a chfr protein, or an anti-sense thereof, and a method of determining tumorigenic potential of a cell comprising examining the presence of chfr nucleic acid sequence.

Group II, claim(s) 7-10, drawn to a chfr protein.

Group III, claim(s) 14-15, drawn to a method of determining tumorigenic potential of a cell comprising examining the presence of chfr.

Group IV, claims 16-19, drawn to a method of determining tumorigenic potential of a cell comprising examining the presence of mutation in chfr gene.

Group V, claim 20, drawn to a method of determining tumorigenic potential of a cell comprising examining chfr-mediated ubiquitin-protein ligase activity.

Group VI, claims 23-25, 27-30, 32, 38, drawn to a ligand, or an inhibitor of chfr.

Group VII, claim 26, drawn to a kit for assaying a chfr-mediated ubiquitin-protein ligase activity.

Group VIII, claims 31, 35-37, drawn to a method for identifying a chfr inhibitor.

Group IX, claims 39-41, drawn to a method for retarding tumor cell growth.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

Group I, species SEQ ID NO:1 or an antisense sequence thereof.

Group VI, species antibodies or chemical compounds.

The invention listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.1, they lack the same or corresponding special technical features for the following reasons:

An international stage application shall relate to one invention only or to a group of invention so linked as to form a single general inventive concept. If multiple products, processes of manufacture or uses are claimed, the first invention of the category first mentioned in the claims of the application will be considered as the main invention in the claims, see PCT article 17(3) (a) and 1.476 (c), 37 C.F.R. 1.475(d). Group I will be the main invention. After that, all other products and methods will be broken out as separate groups (see 37 CFR 1.475 (d)).

Group I, drawn to a nucleic acid sequence encoding chfr protein, and a method of use, forms a single inventive concept.

Although SEQ ID NO:1 is not known in the art, a chfr gene is known in the art, and has the same properties as the claimed chfr gene in claim 1 (Scolnick et al, Proceed. Amer. Assoc. Cancer Res, March 1999, Vol 40, page 214, abstract No: 1422). Therefore, chfr would not be a special technical feature to link different groups.

Group II is an additional product, a chfr protein, which is structurally distinct from the nucleic acid sequence of group I.

Groups III, IV and V are additional methods of use, which are different from the method of group I, because each method uses different means for determining the tumorigenic potential of a cell.

Group VI is an additional product, a ligand, which is structurally distinct from the nucleic acid sequence of group I.

Group VII is an additional product, components for an enzyme assay, which are structurally distinct from the nucleic acid sequence of group I.

Groups VIII and IX are additional methods of use, which have different objectives, and use different reagents than the method of group I.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The species SEQ ID NO:1 and its antisense sequences of group I are distinct, because the structure of said antisense sequence is unpredictable even if SEQ ID NO:1 is known.

The species antibodies and chemical compounds of group VI are distinct, because they are structurally different from each other.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 February 2001 (08.02.2001)

PCT

(10) International Publication Number
WO 01/09150 A2

- (51) International Patent Classification⁷: C07H Thanos [US/US]; 765 Periwinkle Lane, Wynnewood, PA 19096 (US). SCOLNICK, Daniel [US/US]; Apartment A210, 701 City Line Avenue, Merion, PA 19066 (US).
- (21) International Application Number: PCT/US00/16391
- (22) International Filing Date: 14 June 2000 (14.06.2000) (74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).
- (25) Filing Language: English
- (26) Publication Language: English
- (81) Designated States (*national*): AU, CA, JP, US.
- (30) Priority Data: 60/146,194 29 July 1999 (29.07.1999) US (84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
- (71) Applicant (*for all designated States except US*): THE WISTAR INSTITUTE OF ANATOMY & BIOLOGY [US/US]; 3601 Spruce Street, Philadelphia, PA 19104-4268 (US). Published:
--- Without international search report and to be republished upon receipt of that report.
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): HALAZONETIS,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITIONS AND METHODS TO ENHANCE SENSITIVITY OF CANCER CELLS TO MITOTIC STRESS

(57) Abstract: An isolated nucleic acid sequence of a mitotic checkpoint gene, *chfr*, encodes a Chfr protein having a Forkhead-associated domain and a Ring Finger. This protein is required for regulation of the transition of cells from prophase to metaphase during mitosis. The *chfr* nucleic acid and Chfr polypeptide are useful in diagnosing tumorigenic cells and in screening for drugs which can inhibit the activity of Chfr in a cancer cell, thereby rendering the cell more sensitive to additional anti-tumor therapies.



WO 01/09150 A2

PATENT COOPERATION TREATY



00270

PATENT TRADEMARK OFFICE

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

WRITTEN OPINION

(PCT Rule 66)

To: MARY E. BAK
HOWSON AND HOWSON
SPRING HOUSE CORPORATE CENTER
P.O. BOX 457
SPRING HOUSE, PENNSYLVANIA 19477

Date of Mailing
(day/month/year) **28 FEB 2001**

Applicant's or agent's file reference

WS197APCT

REPLY DUE within **ONE** months
from the above date of mailing

International application No.

PCT/US00/16391

International filing date (day/month/year)

14 JUNE 2000

Priority date (day/month/year)

29 JULY 1999

International Patent Classification (IPC) or both national classification and IPC
Please See Supplemental Sheet.

Applicant

THE WISTAR INSTITUTE OF ANATOMY & BIOLOGY

1-28-02

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority

2. This opinion contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 66.2 is 29 NOVEMBER 2001

Name and mailing address of the IPEA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Jayne Bridges for
MISH TAM DAVIS

Telephone No. (703) 308-0916

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

- ☒ the international application as originally filed
- ☒ the description:
pages 1-47 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____
- ☒ the claims:
pages 48-55 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____
- ☒ the drawings:
pages 1-12 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____
- ☒ the sequence listing part of the description:
pages 1-17 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23 1(b))
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

- ☐ contained in the international application in printed form
- ☐ filed together with the international application in computer readable form
- ☒ furnished subsequently to this Authority in written form
- ☒ furnished subsequently to this Authority in computer readable form
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4 ☒ The amendments have resulted in the cancellation of

- ☒ the description, pages NONE
- ☒ the claims, Nos. NONE
- ☒ the drawings, sheets/fig NONE

5 ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70 2(e))

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 14-20,26,28-29,31,33-37,39-42

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination (*specify*).

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. _ are so unclear that no meaningful opinion could be formed (*specify*).

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. _ (*See Attached*).

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions

☐ the written form has not been furnished or does not comply with the standard

☐ the computer readable form has not been furnished or does not comply with the standard

IV. Lack of unity of invention

1. In response to the invitation (Form PCT/ISA 405) to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
- ☐ paid additional fees.
- ☐ paid additional fees under protest.
- ☒ neither restricted nor paid additional fees.

2. This Authority found that the requirement of unity of invention is not complied with for the following reasons and chose, according to Rule 68.1 not to invite the applicant to restrict or pay additional fees:

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

- ☐ all parts.
- ☒ the parts relating to claims Nos. 1-13, 21-25, 27, 30, 32, 38.

WRITTEN OPINION

International application No

PCT/US00/16391

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)

Claims 2-13,21-25,27,30,32,38 YES
Claims 1 NO

Inventive Step (IS)

Claims 2-13,21-25,27,30,32,38 YES
Claims 1 NO

Industrial Applicability (IA)

Claims 1-13,21-25,27,30,32,38 YES
Claims NONE NO

2. citations and explanations

Claim 1 lacks novelty under PCT Article 33(2) as being anticipated by Scolnick et al.

Scolnick et al teach a chfr gene that is involved in the spindle checkpoint, that delays the condensing of the chromosome. Thus the gene taught by the art is the same as the claimed chfr gene.

Claims 2-13,21-25,27,30,32,38 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a polypeptide of SEQ ID NO:2. Further, since the sequence structure of the chfr gene is not taught in the art, the methods of use of said gene and the encoded polypeptide are not obvious.

----- NEW CITATIONS -----
NONE

WRITTEN OPINION

International application No.

PCT/US00/16391

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C07H 21/04; C07K 14/00; C12Q 1/68; G01N 33/53; A61K 39/395 and US Cl.: 536/23.1; 530/350; 435/6, 7, 1; 424/130.1

III. NON-ESTABLISHMENT OF OPINION:

No international search report has been established for claim numbers 14-21,26,28-29,31,33-37,39-42.



The demand must be filed with the competent International Preliminary Examining Authority or, if two or more authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority must be indicated by the applicant on the line below:

IPEA/ US

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only	
Identification of IPEA	Date of receipt of DEMAND
Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION	
Applicant's or agent's file reference WST97APCT	
International application No. PCT/US00/16391	International filing date (day/month/year) 14 June 2000 (14.06.00)
(Earliest) Priority date (day/month/year) 29 July 1999 (29.07.99)	
Title of invention COMPOSITIONS AND METHODS TO ENHANCE SENSITIVITY OF CANCER CELLS TO MITOTIC STRESS	
Box No. II APPLICANT(S)	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) The Wistar Institute of Anatomy & Biology 3601 Spruce Street Philadelphia, Pennsylvania 19104-4268 United States	
Telephone No.: 215-898-0049	
Facsimile No.: 215-573-2456	
Teleprinter No.:	
State (that is, country) of nationality: US	State (that is, country) of residence: US
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) Halazonetis, Thanos 765 Periwinkle Lane Wynnewood, Pennsylvania 19096 United States	
State (that is, country) of nationality: US	State (that is, country) of residence: US
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) Scolnick, Daniel 701 City Line Avenue Apartment A210 Merion, Pennsylvania 19066 United States	
State (that is, country) of nationality: US	State (that is, country) of residence: US
<input type="checkbox"/> Further applicants are indicated on a continuation sheet.	

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative
 and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.
☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.
☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation.
The address must include postal code and name of country.)*

Bak, Mary E.
 Howson and Howson
 Spring House Corporate Center
 P.O. Box 457
 Spring House, Pennsylvania 19477
 United States

Telephone No.:

215-540-9200

Facsimile No.:

215-540-5818

Teleprinter No.:

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION**Statement concerning amendments: ***

1. The applicant wishes the international preliminary examination to start on the basis of:

- ☒ the international application as originally filed
- the description ☐ as originally filed
☐ as amended under Article 34
- the claims ☐ as originally filed
☐ as amended under Article 19 (together with any accompanying statement)
☐ as amended under Article 34
- the drawings ☐ as originally filed
☐ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

- ☒ which is the language in which the international application was filed.
☐ which is the language of a translation furnished for the purposes of international search.
☐ which is the language of publication of the international application.
☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States (that is, all States which have been designated and which are bound by Chapter II of the PCT)

excluding the following States which the applicant wishes not to elect:

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:

- | | | |
|--|---|--------|
| 1. translation of international application | : | sheets |
| 2. amendments under Article 34 | : | sheets |
| 3. copy (or, where required, translation) of amendments under Article 19 | : | sheets |
| 4. copy (or, where required, translation) of statement under Article 19 | : | sheets |
| 5. letter | : | sheets |
| 6. other (specify) | : | sheets |

For International Preliminary
Examining Authority use only

received not received

<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>

The demand is also accompanied by the item(s) marked below:

- | | |
|--|---|
| 1. <input checked="" type="checkbox"/> fee calculation sheet | 4. <input type="checkbox"/> statement explaining lack of signature |
| 2. <input type="checkbox"/> separate signed power of attorney | 5. <input type="checkbox"/> nucleotide and or amino acid sequence listing in computer readable form |
| 3. <input type="checkbox"/> copy of general power of attorney; reference number, if any: | 6. <input type="checkbox"/> other (specify): |

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

By: Mary E. Bak
Mary E. Bak
Attorney for Applicants

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b).

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply.

☐ The applicant has been informed accordingly.

4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.

5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.



00270

OFFICE RECEIVING OFFICE

International Application No.
International Filing Date
Name of receiving Office and "PCT International Application"
Applicant's or agent's file reference (if desired) (12 characters maximum) WST97APCT

Box No. I TITLE OF INVENTION
COMPOSITIONS AND METHODS TO ENHANCE SENSITIVITY OF CANCER CELLS TO MITOTIC STRESS

Box No. II APPLICANT	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) The Wistar Institute of Anatomy & Biology 3601 Spruce Street Philadelphia, PA 19104-4268 United States	<input type="checkbox"/> This person is also inventor Telephone No. 215-898-0049 Facsimile No. 215-573-2456 Teleprinter No.
State (that is, country) of nationality: United States	State (that is, country) of residence: United States
This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input checked="" type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box	

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) Halazonetis, Thanos 765 Periwinkle Lane Wynnewood, Pennsylvania 19096 United States	This person is: <input type="checkbox"/> applicant only <input checked="" type="checkbox"/> applicant and inventor <input type="checkbox"/> inventor only (if this check-box is marked, do not fill in below)
State (that is, country) of nationality: United States	State (that is, country) of residence: United States
This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box	
<input checked="" type="checkbox"/> Further applicants and/or (further) inventors are indicated on a continuation sheet.	

Box No. IV AGENT OR COMMON REPRESENTATIVE: OR ADDRESS FOR CORRESPONDENCE	
The person identified below is hereby has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: <input checked="" type="checkbox"/> agent <input type="checkbox"/> common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) Bak, Mary E. Howson and Howson Spring House Corporate Center P.O. Box 457 Spring House, Pennsylvania 19477 United States	Telephone No. 215-540-9200 Facsimile No. 215-540-5818 Teleprinter No.
<input type="checkbox"/> Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
<i>If none of the following sub-boxes is used, this sheet should not be included in the request.</i>	
<p>Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i></p> <p>Scolnick, Daniel 701 City Line Avenue Apartment A210 Merion, Pennsylvania 19066 United States</p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input checked="" type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i></p>
State <i>(that is, country)</i> of nationality: United States	State <i>(that is, country)</i> of residence: United States
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i></p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i></p>
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i></p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i></p>
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i></p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i></p>
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p><input type="checkbox"/> Further applicants and/or (further) inventors are indicated on another continuation sheet.</p>	

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

Regional Patent

- ☐ **AP** ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☐ **EA** Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP** European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☐ **OA** OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)

National Patent (if other kind of protection or treatment desired, specify on dotted line):

- | | |
|--|--|
| <input type="checkbox"/> AE United Arab Emirates | <input type="checkbox"/> LR Liberia |
| <input type="checkbox"/> AL Albania | <input type="checkbox"/> LS Lesotho |
| <input type="checkbox"/> AM Armenia | <input type="checkbox"/> LT Lithuania |
| <input type="checkbox"/> AT Austria | <input type="checkbox"/> LU Luxembourg |
| <input checked="" type="checkbox"/> AU Australia | <input type="checkbox"/> LV Latvia |
| <input type="checkbox"/> AZ Azerbaijan | <input type="checkbox"/> MA Morocco |
| <input type="checkbox"/> BA Bosnia and Herzegovina | <input type="checkbox"/> MD Republic of Moldova |
| <input type="checkbox"/> BB Barbados | <input type="checkbox"/> MG Madagascar |
| <input type="checkbox"/> BG Bulgaria | <input type="checkbox"/> MK The former Yugoslav Republic of Macedonia |
| <input type="checkbox"/> BR Brazil | <input type="checkbox"/> MN Mongolia |
| <input type="checkbox"/> BY Belarus | <input type="checkbox"/> MW Malawi |
| <input checked="" type="checkbox"/> CA Canada | <input type="checkbox"/> MX Mexico |
| <input type="checkbox"/> CH and LI Switzerland and Liechtenstein | <input type="checkbox"/> NO Norway |
| <input type="checkbox"/> CN China | <input type="checkbox"/> NZ New Zealand |
| <input type="checkbox"/> CR Costa Rica | <input type="checkbox"/> PL Poland |
| <input type="checkbox"/> CU Cuba | <input type="checkbox"/> PT Portugal |
| <input type="checkbox"/> CZ Czech Republic | <input type="checkbox"/> RO Romania |
| <input type="checkbox"/> DE Germany | <input type="checkbox"/> RU Russian Federation |
| <input type="checkbox"/> DK Denmark | <input type="checkbox"/> SD Sudan |
| <input type="checkbox"/> DM Dominica | <input type="checkbox"/> SE Sweden |
| <input type="checkbox"/> EE Estonia | <input type="checkbox"/> SG Singapore |
| <input type="checkbox"/> ES Spain | <input type="checkbox"/> SI Slovenia |
| <input type="checkbox"/> FI Finland | <input type="checkbox"/> SK Slovakia |
| <input type="checkbox"/> GB United Kingdom | <input type="checkbox"/> SL Sierra Leone |
| <input type="checkbox"/> GD Grenada | <input type="checkbox"/> TJ Tajikistan |
| <input type="checkbox"/> GE Georgia | <input type="checkbox"/> TM Turkmenistan |
| <input type="checkbox"/> GH Ghana | <input type="checkbox"/> TR Turkey |
| <input type="checkbox"/> GM Gambia | <input type="checkbox"/> TT Trinidad and Tobago |
| <input type="checkbox"/> HR Croatia | <input type="checkbox"/> TZ United Republic of Tanzania |
| <input type="checkbox"/> HU Hungary | <input type="checkbox"/> UA Ukraine |
| <input type="checkbox"/> ID Indonesia | <input type="checkbox"/> UG Uganda |
| <input type="checkbox"/> IL Israel | <input checked="" type="checkbox"/> US United States of America |
| <input type="checkbox"/> IN India | <input type="checkbox"/> UZ Uzbekistan |
| <input type="checkbox"/> IS Iceland | <input type="checkbox"/> VN Viet Nam |
| <input checked="" type="checkbox"/> JP Japan | <input type="checkbox"/> YU Yugoslavia |
| <input type="checkbox"/> KE Kenya | <input type="checkbox"/> ZA South Africa |
| <input type="checkbox"/> KG Kyrgyzstan | <input type="checkbox"/> ZW Zimbabwe |
| <input type="checkbox"/> KP Democratic People's Republic of Korea | |
| <input type="checkbox"/> KR Republic of Korea | |
| <input type="checkbox"/> KZ Kazakhstan | |
| <input type="checkbox"/> LC Saint Lucia | |
| <input type="checkbox"/> LK Sri Lanka | |

Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.3(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)

See Notes to the request form

Supplemental Box

If the Supplemental Box is not used, this sheet should not be included in the request.

1. If, in any of the Boxes, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:

- (i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;
- (ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;
- (iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;
- (iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;
- (v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "continuation" or "continuation-in-part": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;
- (vi) if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;
- (vii) if, in Box No. VI, the earlier application is an ARIPO application: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed.

2. If, with regard to the precautionary designation statement contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.

3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.

"Continuation of Box No. IV"

Kita, Stanley B.
Smith, George A., Jr.
Kodroff, Cathy A.
Bak, William

All above attorneys are members of the firm of Howson and Howson.
Address of all is indicated in Box No. IV.

See Notes to the request form

Box No. VI PRIORITY CLAIM		<input type="checkbox"/> Further priority claims are indicated in the Supplemental Box.		
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application: regional Office	international application: receiving Office
item (1) 29 July 1999	60/146,194	US		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s). (1)

* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA)
(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):

ISA / US

Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):

Date (day/month/year)

Number

Country (or regional Office)

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following number of sheets:

request : 5
description (excluding sequence listing part) : 47
claims : 8
abstract : 1
drawings : 12
sequence listing part of description : 17

Total number of sheets : 90

This international application is accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other (specify): transmittal letter to the USRO

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

By: Mary E. Bak
Mary E. Bak
Attorney for Applicants

For receiving Office use only		2. Drawings: <input type="checkbox"/> received: <input type="checkbox"/> not received
1. Date of actual receipt of the purported international application:		
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application		
4. Date of timely receipt of the required corrections under PCT Article 11(2)		
5. International Searching Authority (if two or more are competent): ISA /	6. <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid.	

For International Bureau use only	
Date of receipt of the record copy by the International Bureau:	



00270

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 February 2001 (08.02.2001)

PCT

(10) International Publication Number
WO 01/09150 A3

- (51) International Patent Classification⁷: C07H 21/04, 19096 (US). SCOLNICK, Daniel [US/US]; Apartment C07K 16/00 A210, 701 City Line Avenue, Merion, PA 19066 (US).
- (21) International Application Number: PCT/US00/16391 (74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).
- (22) International Filing Date: 14 June 2000 (14.06.2000)
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(54) Title: COMPOSITIONS AND METHODS TO ENHANCE SENSITIVITY OF CANCER CELLS TO MITOTIC STRESS

(57) Abstract: An isolated nucleic acid sequence of a mitotic checkpoint gene, *chfr*, encodes a Chfr protein having a Forkhead-associated domain and a Ring Finger. This protein is required for regulation of the transition of cells from prophase to metaphase during mitosis. The *chfr* nucleic acid and Chfr polypeptide are useful in diagnosing tumorigenic cells and in screening for drugs which can inhibit the activity of Chfr in a cancer cell, thereby rendering the cell more sensitive to additional anti-tumor therapies.

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COMPOSITIONS AND METHODS TO ENHANCE SENSITIVITY OF CANCER CELLS TO MITOTIC STRESS

5 **Field of the Invention**

This invention relates generally to a novel gene, proteins encoded thereby, compositions containing same and methods of use therefor. More specifically, this invention relates to a novel cell cycle gene, and its uses in diagnosis and drug screening.

10

Background of the Invention

Several critical processes occur during the four stages of mitotic cell division, which are prophase, metaphase, anaphase and telophase, including, without limitation, separation of the centrosomes and preparation of the cell to form the mitotic spindle; alignment of the chromosomes on the spindle in metaphase; and sister chromatid separation in anaphase. Specifically, during prophase the duplicated centrosomes migrate along the periphery of the nucleus towards opposite poles of the cell. During prophase the cell may also prepare for chromosome condensation and for other events that occur in metaphase. A critical and irreversible event during the transition from metaphase to anaphase is the irreversible segregation of sister chromatids between daughter cells.

The fidelity of mitosis is monitored by checkpoint genes. For example, a multitude of evolutionarily conserved checkpoint genes monitor the metaphase to anaphase transition. Several of these checkpoint genes have been identified, initially in yeast, and later in higher eukaryotes, that prevent the onset of anaphase until the mitotic spindle is properly assembled [Elledge, 1998, Science, 279:999-1000; Amon, 1999, Curr. Opin. Genetics Dev., 9:69-75]. The presence of these checkpoint genes, coupled with the predisposition towards aneuploidy when these checkpoint genes are inactivated, provide evidence that this transition is clearly an important milestone for mitosis. Although most of the research on mitotic checkpoints has focused on the spindle checkpoint, which monitors the transition from metaphase to anaphase, given

the complexity of the mitotic process, the existence of additional checkpoints that monitor other phases of mitosis is likely. A checkpoint monitoring the anaphase-to-telophase transition has been described [Muhua, L. *et al*, 1998 Nature 393: 487-491].

Errors during mitosis can result in unequal chromosome segregation and are probably responsible for the aneuploid phenotype of cancer cells. Agents that target microtubules induce mitotic stress and thus cause such errors [McIntosh, J.R. & Koonce, M.P., 1989 Science, 246:622-628, Jordan, M.A. & Wilson, L., 1998 Curr. Opin. Cell Biol., 10: 123-130]. Many human cancers are sensitive to mitotic stress. This sensitivity is being exploited for therapy and implies that tumor cells have mitotic checkpoint defects [Lengauer *et al*, 1998, Nature, 396:643-649; Hartwell, L.H. & Kastan, M.B., 1994 Science, 266:1821-1828; Lengauer, C. *et al*, 1997 Nature 386:623-627; Lengauer, C. *et al*, 1998 Nature 396:643-649; Elledge, S.J. 1998 Science 279: 999-1000; Amon, A. 1999 Curr. Opin. Genet. Dev. 9: 69-75; and Li, Y. & Benezra, R., 1996 Science, 274: 246-248]. However, the known mitotic checkpoint genes, which prevent entry into anaphase when the chromosomes are not properly aligned on the mitotic spindle, are rarely inactivated in human cancer [Yamaguchi, K. *et al*, 1999 Cancer Lett. 139:183-187; Jin, D.Y. *et al*, 1998 Cell 93:81-91; Zou, H. *et al*, 1999 Science 285, 418-422]. For example, many of the mitotic spindle checkpoint genes have been examined for mutations in human cancer, but so far only infrequent *bub 1* mutations have been detected [Cahill *et al*, 1998, Nature, 392:300-303; Cahill *et al*, 1999, Genomics, 58:181-187]. Thus, the molecular basis of cancer aneuploidy remains elusive, except for the small number of cases with *bub 1* mutations.

Thus, there remains a need in the art for the identification of additional methods and compositions useful in the diagnosis of cancer, particularly the identification of additional genes that monitor and control mitosis, as well as methods and compositions that permit the screening of drugs useful for treatment of cancer. The present invention satisfies this need.

Summary of the Invention

In one aspect, the invention provides an isolated nucleic acid sequence of a mitotic checkpoint gene, *chfr*, which encodes a Chfr protein having a Forkhead-associated (FHA) domain and a Ring Finger (RF) domain. The protein is required for regulation of the transition of cells from prophase to metaphase during mitosis.

In another aspect, the invention provides a substantially pure preparation of a polypeptide comprising an FHA domain and an RF domain. This protein is required for regulation of the transition of a normal human cell from prophase to metaphase during mitosis.

In still another aspect, the invention provides a method of determining tumorigenic potential of a cell comprising examining the cell for the presence of *chfr* nucleic acid sequence in the cell, wherein the absence of the *chfr* nucleic acid sequence indicates that the cell is sensitive to mitotic stress.

In yet another aspect, the invention provides a method of determining the tumorigenic potential of a cell comprising examining the cell for the presence of Chfr polypeptide expression in the cell, wherein the absence of the polypeptide sequence indicates that the cell is sensitive to mitotic stress.

In still another aspect, the invention provides a method for determining tumorigenic potential of a cell comprising examining the cell for mutations in the *chfr* gene, wherein the presence of mutations in the gene indicates that the cell is predisposed to tumorigenesis upon exposure to mitotic stress.

In another aspect, the invention provides a method for determining tumorigenic potential of a cell comprising examining the cell for Chfr-mediated ubiquitin-protein ligase activity, wherein the absence of this activity indicates that the cell is predisposed to tumorigenesis upon exposure to mitotic stress.

In a further aspect, the invention provides a diagnostic reagent comprising a nucleotide sequence that binds to the *chfr* nucleic acid sequence or a fragment thereof. The reagent sequence is preferably associated with a detectable label.

In still another aspect, the invention provides a diagnostic reagent comprising a ligand which binds to Chfr, the ligand associated with a detectable label.

Yet another aspect of this invention is a diagnostic kit for detecting the sensitivity of a cell to mitotic stress. The kit comprises at least one of the above-mentioned diagnostic reagents and suitable components for detection of the label.

5 In yet another aspect, the invention provides a ubiquitin-protein ligase assay useful for determining the activity and/or function of Chfr or screening for a Chfr inhibitor.

In still a further aspect, the invention provides a diagnostic kit for detecting the tumorigenic potential of a cell comprising components for a Chfr-mediated ubiquitin protein ligase assay.

10 In another aspect, the invention provides composition which inhibits the biological activity of Chfr. This inhibitor may be identified by one of the novel methods for identifying such inhibitors described herein.

Thus, in one aspect, a method of identifying a Chfr inhibitor is provided that comprises the steps of: (a) contacting a cell capable of expressing Chfr with a suitable
15 amount of a test compound, and assessing the level of expression of Chfr in the cell; (b) assessing the level of expression of Chfr in an otherwise identical cell which has not been contacted with the test compound; and (c) comparing the levels of Chfr expression. A lower level of expression of the Chfr in the cell (a) compared with the level of Chfr in the cell (b) indicates that the test compound is a Chfr inhibitor.

20 In another aspect, the invention provides a method of identifying a Chfr inhibitor that comprises screening a test compound in a Chfr-mediated ubiquitin-protein ligase assay, wherein the substantial absence of, or reduction in, the ligase activity in the assay in the presence of the test compound indicates that the test compound inhibits Chfr function. This assay may involve contacting a mixture which
25 normally demonstrates Chfr-mediated ubiquitin-protein ligase activity with a test compound, and assaying the mixture and test compound for the activity. The substantial absence of the activity in the presence of the test compound indicates that the test compound inhibits Chfr function.

30 In still a further aspect, the invention provides a method of retarding the growth of a cancer cell, the method comprising administering to the cell a Chfr

inhibitor that enhances the sensitivity of the cell to mitotic stress. This method may be performed *in vivo* by direct administration to the mammal.

In still another aspect, a method of assessing the sensitivity of a tumor cell to an agent which disrupts microtubule function includes the steps of examining the cell for at least one of the following characteristics: the substantial absence of a *chfr* gene; the substantial absence of Chfr protein; the substantial absence of Chfr-mediated ubiquitin-protein ligase activity; and a mutation in the *chfr* gene. The identification of any of these characteristics provides an indication that the tumor cell is sensitive to an agent which disrupts microtubule function. The specific assay steps used in the determination are described herein.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1A is a schematic illustrating the structural domains of human Chfr (Chfr_hs), *S. pombe* Dma1 (Dma1_sp), the *S. cerevisiae* predicted open reading frames YHR115c (YHR115c_sc) and YNL116w (YNL115c_sc). The FHA domain, the RF domain and the cysteine-rich (CR) region are indicated. The numbers refer to amino acid positions.

Fig. 1B illustrates the alignments of the FHA domains of *S. cerevisiae* Rad53 [SEQ ID NO:3], human chfr amino acids 31-103 of SEQ ID NO: 2, *S. pombe* Dma 1 [SEQ ID NO: 4], and the *S. cerevisiae* predicted open reading frame YNL116w [SEQ ID NO: 5]. The consensus (cons.) sequence of the FHA domains is also indicated.

Fig. 1C illustrates the alignments of the ring finger domains of the *Varicella zoster* virus ICP0 [SEQ ID NO: 6], human chfr amino acids 303 to 346 of SEQ ID NO: 2, *S. pombe* Dma 1 [SEQ ID NO:7], and *S. cerevisiae* predicted open reading frame YNL116w [SEQ ID NO:8]. The consensus (cons.) sequence of the RF domains is also indicated.

Fig. 2 is a graph illustrating the mitotic index of unsynchronized human tumor cell lines exposed to nocodazole for 16 hours, demonstrating the fact that chfr

regulates the response of cells to mitotic stress. The names of the cell lines that do not express *chfr* are underlined.

Fig. 3A illustrates the double-stranded nucleotide sequence of wild-type (wt) *chfr* [SEQ ID NO: 1] encoding amino acid residues Leu₅₇₉ Val₅₈₀ Ala₅₈₁ of SEQ ID NO: 2. The dinucleotide CG in the non coding strand is underlined and italicized. L, Leu; V, Val; A, Ala.

Fig. 3B illustrates the double-stranded nucleotide sequence of variant *chfr* cDNA from U20S cells [SEQ ID NO: 1] corresponding to amino acid residues 579-581 which bear a *chfr* missense mutation. The U20S sequence Leu₅₇₉ Met₅₈₀ Ala₅₈₁ of SEQ ID NO: 2 shows Met₅₈₀, not Val₅₈₀ as in wildtype. The relevant codon is underlined. The mutated dinucleotide mutation TG is underlined and italicized. L, Leu; M, Met; A, Ala.

Fig. 3C is a bar graph depicting the mitotic index of unsynchronized U20S and DLD1 cells transiently-transfected with plasmids expressing wild-type (wt) or mutant (Met₅₈₀) *chfr* in response to nocodazole treatment for 16 hours.

Figs. 4A-4D illustrates the continuous *chfr* nucleotide sequence [SEQ ID NO:1], as well as the continuous amino acid sequence of Chfr [SEQ ID NO:2].

Fig. 5 is a bar graph showing the "long-term" response of synchronized DLD1-neo and DLD1-*chfr* cells exposed transiently to mitotic stress, e.g., to nocodazole (Noc) or taxol (T) 12 hours after release from the G1-S block for a 4 hour period. The cells were replated and scored for colony formation 3 weeks later. The controls are indicated by (-).

Fig. 6 is a bar graph showing the mitotic index of unsynchronized DLD1 cells stably transfected with plasmids expressing neo or wild-type Chfr or Chfr-A₃₂₅ after exposure to nocodazole for 16 hours.

Fig. 7A is a bar graph illustrating the mitotic index of unsynchronized U20S and DLD 1 cells stably transfected with plasmids expressing neo or wildtype *chfr* or *chfr*-M₅₈₀ after exposure to nocodazole for 16 hours.

Fig. 7B is a bar graph showing mitotic index of unsynchronized SAOS2 cells transiently-transfected with plasmids expressing no Chfr protein (vec), wild-type Chfr

or Chfr Δ FHA. Taxol was added 36 hours after the transient transfection and the mitotic index was determined 8 (white bar), 12 (gray bar), 14 (first black bar) and 16 (second black bar) hours later.

Fig. 7C is a bar graph illustrating the mitotic index of unsynchronized DLD 1 cells transiently-transfected with plasmids expressing no Chfr protein (DLD1-vec), wild-type Chfr (DLD-chfr; 1 μ g), ChfrMet₅₈₀ (DLD1-M₅₈₀; 5 μ g), Chfr Δ FHA (DLD1- Δ FHA; 5 μ g), or wild-type Chfr (1 μ g) and ChfrM₅₈₀ (5 μ g) (DLD1-chfr+M₅₈₀), or wild-type Chfr (1 μ g) and Chfr Δ FHA (5 μ g) (DLD1-chfr- Δ FHA). Taxol was added 36 hours after the transient transfection and the mitotic index was determined 16 hours later.

Fig. 8A is a graph showing mitotic index of synchronized DLD1 cells stably-transfected with plasmids expressing neo (DLD1-neo) as a function of time in hours after release from the G1-S block. The cells were either not exposed to mitotic stress (\square) or treated with nocodazole (\blacksquare), taxol (\bullet) or colcemid (\blacklozenge) 12 hours after release from the cell cycle block or treated with nocodazole (\times) 14 hours after release.

Fig. 8B is a graph showing mitotic index of synchronized DLD1 cells stably-transfected with plasmids expressing chfr (DLD1-chfr) as a function of time after release from the G1-S block. The cells were treated as described for Fig. 8A (symbols are identical).

Fig. 8C is a graph showing mitotic index of synchronized normal (primary) human epidermal keratinocytes in the absence (\square) and presence of mitotic stress induced with nocodazole, N, 12 hours after release from the G1-S block (\blacklozenge).

Fig. 8D is a graph showing mitotic index of synchronized normal (primary) human osteoblasts in the absence (\square) and presence of mitotic stress induced by taxol, T, (\bullet) or nocodazole, N, (\blacklozenge) 6 hours after release from the G1-S block.

Detailed Description of the Invention

The invention relates to the discovery of a novel gene that functions as a mitotic checkpoint, and to the uses of the gene and the protein expressed therefrom in diagnostic, therapeutic and drug-screening applications.

A. *The chfr Gene and Chfr Polypeptide*

The novel mitotic checkpoint gene of this invention, referred to as *chfr*, is characterized by the presence of a ForkHead-Associated (FHA) DNA-binding domain and a ring finger motif. FHA domains were initially identified in transcription factors that have forkhead DNA-binding domains and in protein kinases [Hofmann and Bucher, 1995, Trends Bioch. Sci., 20:347-349]. Many proteins that contain FHA domains are currently recognized to be cell cycle checkpoints. Briefly described, the inventors identified this novel gene by screening a database of cDNA sequences for FHA domains. The human gene, hereafter referred to as *chfr*, has the nucleotide sequence reported in Figs. 4A-4D [SEQ ID NO: 1]. The GenBank accession number for human Chfr is AF170724. This gene was noted to have weak similarity to the yeast mitotic checkpoint gene *dmal*. See, Example 1 below. The Chfr polypeptide expressed by this sequence has the amino acid sequence also reported in Figs. 4A-4D [SEQ ID NO: 2]. Therefore, the invention includes an isolated *chfr* nucleic acid and also includes a substantially pure preparation of a Chfr polypeptide.

As disclosed in the Examples 2 and 3 below, Chfr expression is ubiquitous in normal tissues. However, in three of eight human cancer cell lines, *chfr* mRNA and Chfr protein were undetectable. In a fourth human cancer cell line, a missense mutation was identified. The Chfr polypeptide is thereby inactivated due to lack of expression or by mutation in four out of eight examined human cancer cell lines. Normal primary cells, e.g., diploid fibroblasts, and tumor cell lines that express wild-type *chfr* exhibited delayed entry into metaphase (i.e., arrested in prophase) when exposed to an agent which disrupts microtubule function and induces mitotic stress. These agents, such as nocodazole, taxol and colcemid, inhibit centrosome separation. However, the tumor cell lines that have lost *chfr* function passed through prophase, entered metaphase without delay, and arrested in metaphase. Ectopic expression of wild-type *chfr* in these cells restored the cell cycle delay (e.g., prophase arrest) and increased the ability of the cells to survive mitotic stress. As discussed below, nocodazole inhibited centrosome separation, which normally occurs during prophase. Thus, cells that lack *chfr* function entered metaphase despite failure to separate the

centrosomes. Such cells would be expected to have a high frequency of chromosome segregation errors and to survive mitotic stress less well than cells that retain *chfr* function. Thus, *chfr* defines a novel prophase to metaphase transition checkpoint that delays entry into metaphase in response to mitotic stress. A delay in metaphase entry in response to mitotic stress has not been previously described. When *chfr* is inactivated in human cancer cells, the inactivation contributes to aneuploidy and sensitivity to mitotic stress, e.g., such as that caused by agents that disrupt microtubule function or other chemotherapeutic agents.

Thus, in one embodiment, the invention includes an isolated nucleic acid of a *chfr* gene. The term "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, such as the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, e.g., RNA or DNA or proteins, in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., as a cDNA or a genomic fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

The isolated nucleic acid of *chfr* according to this invention should not be construed as being limited solely to the nucleotide sequences presented herein, but rather should be construed to include any and all nucleotide sequences which share homology (i.e., have sequence identity) with the nucleotide sequences presented herein. Preferably, the invention includes an isolated nucleic acid having a nucleotide sequence which is at least 70% identical to the nucleotide sequence presented in Fig. 4A-4D. More preferably, an isolated nucleic acid of this invention has a nucleotide sequence which is at least 75% identical, even more preferably, 80% identical, yet

more preferably, 85% identical, and even more preferably, 90% identical to the nucleotide sequence presented in Figs. 4A-4D. Even more preferably, an isolated nucleic acid of this invention has a nucleotide sequence which is at least 95% identical, and most preferably, 99% identical, to the nucleotide sequence presented in Figs. 4A-4D. Any such isolated nucleic acid would of course encode a polypeptide having the biological activity of the Chfr polypeptide disclosed herein.

"Homologous" as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3' ATTGCC 5' and 3' TATGGC 5' share 50% homology. As used herein, "homology" is used synonymously with "identity".

Percent identity, percent similarity or percent homology of one polynucleotide or polypeptide with respect to another identified polynucleotide or polypeptide may be calculated using algorithms, such as the Smith-Waterman algorithm [J. F. Collins *et al.*, 1988, Comput. Appl. Biosci., 4:67-72; J. F. Collins *et al.*, Molecular Sequence Comparison and Alignment, (M. J. Bishop *et al.*, eds.) In Practical Approach Series: Nucleic Acid and Protein Sequence Analysis XVIII, IRL Press: Oxford, England, UK (1987) pp.417], and the BLAST and FASTA programs [E. G. Shpaer *et al.*, 1996, Genomics, 38:179-191]. A preferred algorithm is the computer program BLAST, especially blastp or tblastn [Altschul *et al.*, 1997 Nucl. Acids Res., 25(17):3389-3402]. These references are incorporated herein by reference. Sequence homology for polypeptides, which is also referred to as sequence identity, is typically

measured using sequence analysis software. See, e.g., the Sequence Analysis Software Package of the Genetics Computer Group (GCG), University of Wisconsin Biotechnology Center, 910 University Avenue, Madison, Wisconsin 53705. Protein analysis software matches similar sequences using a measure of homology assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. For instance, GCG contains programs such as "Gap" and "Bestfit" which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous polypeptides from different species of organisms or between a wild type protein and a mutant thereof. Unless otherwise specified, the parameters of each algorithm discussed above are the default parameters identified by the authors of such algorithms.

Among such homologous nucleotide sequences of this invention are allelic variants of the *chfr* sequences within a species (i.e., sequences containing some individual nucleotide differences from a more commonly occurring sequence within a species, but which nevertheless encode the same polypeptide or a protein with the same function). Additionally nucleic acid sequences capable of hybridizing under stringent conditions [see, J. Sambrook *et al*, Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory (1989)] to the sequences of SEQ ID NO: 1, their anti-sense strands, or biologically active fragments thereof are homologous sequences according to this invention. An example of a highly stringent hybridization condition is hybridization in 2XSSC at 65°C, followed by a washing in 0.1XSSC at 65°C for an hour. Alternatively, an exemplary highly stringent hybridization condition is in 50% formamide, 4XSSC at 42°C. Moderately high stringency conditions may also prove useful, e.g., hybridization in 4XSSC at 55°C, followed by washing in 0.1XSSC at 37°C for an hour. An alternative exemplary moderately high stringency hybridization condition is in 50% formamide, 4XSSC at 30°C.

According to the invention, the *chfr* nucleic acid sequence may be modified. Utilizing the sequence data of SEQ ID NO: 1, it is within the skill of the art to obtain or prepare synthetically or recombinantly other polynucleotide sequences, or modified polynucleotide sequences, encoding the full-length Chfr protein or useful

fragments of the invention. Such modifications at the nucleic acid level include, for example, modifications to the nucleotide sequences which are silent or which change the amino acids, e.g. to improve expression. Also included are allelic variations, caused by the natural degeneracy of the genetic code. Additional homologous sequences can include mutants including 5' or 3' terminal or internal deletions, which truncated or deletion mutant sequence may be expressed for the purpose of affecting the activity of the full-length or wild-type Chfr polypeptide or fragments.

In still another embodiment, the invention provides a substantially pure polypeptide of Chfr. The term "substantially pure" describes a compound, e.g., a protein or polypeptide which has been separated from components which naturally accompany it. Typically, a compound is substantially pure when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, gel electrophoresis or HPLC analysis. A compound, e.g., a protein, is also substantially purified when it is essentially free of naturally associated components or when it is separated from the native contaminants which accompany it in its natural state.

The substantially pure preparation of Chfr according to this invention should not be construed as being limited solely to the amino acid sequences presented herein, but rather should be construed to include any and all amino acid sequences which share homology (i.e., have sequence identity) with the amino acid sequences presented herein. Preferably, the invention includes a polypeptide having an amino acid sequence which is 70% identical, more preferably, 75% identical, even more preferably, 80% identical, yet more preferably, 85% identical, even more preferably, 90% identical, more preferably, 95% identical and most preferably, 99% or 100% identical to the amino acid sequence presented Figs. 4A-4D. This definition of the preparation of Chfr includes the definitions of "homologous", "homology" and "percent identity" as discussed above, including the list of computer algorithms

available to calculate these homologies. Any such preparation of a homologous polypeptide has the biological activity of the Chfr polypeptide disclosed herein.

Also included in the invention are modified versions of the Chfr polypeptide. Typically, such polypeptides differ from the specifically identified Chfr polypeptide of Figs. 4A-4D by only one to four codon changes. Examples include polypeptides with minor amino acid variations from the illustrated partial amino acid sequence of Chfr [SEQ ID NO: 2], in particular, conservative amino acid replacements. Conservative replacements are those that take place within a family of amino acids that are related in their side chains and chemical properties. Further encompassed by this invention are additional fragments of the Chfr polypeptide. These fragments may be designed or obtained in any desired length, including as small as about 5-8 amino acids in length. These small fragments may be useful as probes, primers, molecular weight markers, etc. However, all three fragments, the FHA domain (aa 31-103 of SEQ ID NO: 2), the RF domain (aa 303-346 of SEQ ID NO:2) and the cysteine-rich domain (aa 476 to 641 of SEQ ID NO:2), indicated as black boxes in Fig. 1A, are necessary for Chfr to have biological activity. Fragments of Chfr which are smaller than the full-length Chfr, but which possess these three domains, are desirably characterized by having a biological activity similar to that displayed by the complete Chfr polypeptide, including, e.g., the ability to delay entry into metaphase.

Chfr polypeptides of this invention may be characterized by measurements including, without limitation, western blot, macromolecular mass determinations by biophysical determinations, such as SDS-PAGE/staining, HPLC and the like, and assays such as those in the examples below to identify the biological activity. By the term "biological activity of Chfr" as used herein, is meant the ability to function as a checkpoint between prophase to metaphase in cells wherein in the absence or inactivation of the checkpoint sequence, the cells are predisposed to aneuploidy, and are sensitive to agents which disrupt microtubule function.

B. Methods of Preparing Sequences of this Invention

Methods for obtaining the nucleic acids and polypeptides of the invention should be apparent to those skilled in the art upon a reading of the present disclosure and by following any of the instructions in the art.

5 For example, the nucleotide and polypeptide sequences of the invention may be prepared conventionally by resort to known chemical synthesis techniques, e.g., solid-phase chemical synthesis, such as described by Merrifield, J. Amer. Chem. Soc., 85:2149-2154 (1963), and J. Stuart and J. Young, Solid Phase Peptide Synthesis, Pierce Chemical Company, Rockford, IL (1984), or detailed in the examples below.

10 Alternatively, the nucleotide and polypeptide sequences of this invention may be prepared by known recombinant DNA techniques and genetic engineering techniques, such as polymerase chain reaction, by cloning and expressing within a host microorganism or cell a DNA fragment carrying a nucleic acid sequence encoding the above-described polypeptides, etc. [See, e.g., Sambrook *et al.*, Molecular Cloning. A Laboratory Manual., 2d Edit., Cold Spring Harbor Laboratory, New York
15 (1989); Ausubel *et al.* (1997), Current Protocols in Molecular Biology, John Wiley & Sons, New York]. The Chfr may be obtained from gene banks derived from whole genomic DNA. These sequences, fragments thereof, modifications thereto and the full-length sequences may be constructed recombinantly using conventional molecular
20 biology techniques, site-directed mutagenesis, genetic engineering or PCR, and the like by utilizing the information provided herein. For example, methods for producing the above-identified modifications of the sequences, include mutagenesis of certain nucleotides and/or insertion or deletion of nucleotides, or codons, thereby effecting the polypeptide sequence by insertion or deletion of, e.g., non-natural amino acids, are
25 known and may be selected by one of skill in the art.

1. Expression In Vitro

To produce recombinant Chfr or other fragments of this invention *in vitro* (as well as to produce recombinant proteins of the ubiquitin-protein
ligase assay described herein), the appropriate DNA sequences are inserted into a
30 suitable expression system. Desirably, a recombinant molecule or vector is constructed

in which the polynucleotide sequence encoding the selected protein is operably linked to a heterologous expression control sequence permitting expression of the protein. Numerous types of appropriate expression vectors are known in the art for protein expression, by standard molecular biology techniques. Such vectors are selected from among conventional vector types including insects, e.g., baculovirus expression, or yeast, fungal, bacterial or viral expression systems. Other appropriate expression vectors, of which numerous types are known in the art, can also be used for this purpose. Methods for obtaining such expression vectors are well-known. See, Sambrook *et al*, Molecular Cloning. A Laboratory Manual, 2d edition, Cold Spring Harbor Laboratory, New York (1989); Miller *et al*, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

Suitable host cells or cell lines for transfection by this method include bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, MC1061, and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas*, *Streptomyces*, and other bacilli and the like are also be employed in this method. Mammalian cells, such as human 293 cells, Chinese hamster ovary cells (CHO), the monkey COS-1 cell line or murine 3T3 cells derived from Swiss, Balb-c or NIH mice are used. Another suitable mammalian cell line is the CV-1 cell line. Still other suitable mammalian host cells, as well as methods for transfection, culture, amplification, screening, production, and purification are known in the art. [See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman *et al*, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley *et al*, U. S. Patent 4,419,446]. Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Other fungal cells may also be employed as expression systems. Alternatively, insect cells such as *Spodoptera frugiperda* (Sf9) cells may be used.

Thus, the present invention provides a method for producing a recombinant *Chfr* protein, which involves transfecting, e.g., by conventional means such as electroporation, a host cell with at least one expression vector containing a

polynucleotide of the invention under the control of a transcriptional regulatory sequence. The transfected or transformed host cell is then cultured under conditions that allow expression of the protein. The expressed protein is recovered, isolated, and optionally purified from the cell (or from the culture medium, if expressed extracellularly) by appropriate means known to one of skill in the art. For example, the proteins are isolated in soluble form following cell lysis, or extracted using known techniques, e.g., in guanidine chloride. If desired, the proteins or fragments of the invention are produced as a fusion protein to enhance expression of the protein in a selected host cell, to improve purification, or for use in monitoring the presence of the desired protein in tissues, cells or cell extracts. Suitable fusion partners for the proteins of the invention are well known to those of skill in the art and include, among others, β -galactosidase, glutathione-S-transferase, and poly-histidine.

2. *Expression In Vivo*

Alternatively, where it is desired that the Chfr protein of the invention or proteinaceous inhibitors thereof (whether full-length or a desirable fragment) be expressed *in vivo*, e.g., to induce antibodies, or as a therapeutic, an appropriate vector for delivery is readily selected by one of skill in the art. Exemplary vectors for *in vivo* gene delivery are readily available from a variety of academic and commercial sources, and include, e.g., adeno-associated virus [International patent application No. PCT/US91/03440], adenovirus vectors [M. Kay *et al*, Proc. Natl. Acad. Sci. USA, 91:2353 (1994); S. Ishibashi *et al*, J. Clin. Invest., 92:883 (1993)], or other viral vectors, e.g., various poxviruses, vaccinia, etc. Methods for insertion of a desired gene, e.g., P7-1, and obtaining *in vivo* expression of the encoded protein, are well known to those of skill in the art.

The preparation or synthesis of the nucleotide and polypeptide sequences disclosed herein, whether *in vitro* or *in vivo* (including *ex vivo*) is well within the ability of the person having ordinary skill in the art using available material. The synthetic methods are not a limitation of this invention.

*C. Inhibitors of chfr or Chfr of the Invention and Compositions
Containing Them*

In still another embodiment, the invention provide inhibitors of the *chfr* gene or Chfr polypeptide. Such inhibitor compositions have utility as diagnostic reagents or as therapeutic reagents in the methods described below. By the use of the term "*chfr* inhibitor" as used herein is meant a compound which is capable of inhibiting expression and or biological activity of Chfr. Inhibition of Chfr activity, function or expression may be assessed by following the procedures presented in the examples herein, which permit the progress (or the lack thereof) of a cell from prophase to metaphase to be monitored.

I. Nucleotide sequence inhibitors

One such inhibitor is a nucleotide sequence that binds to the *chfr* nucleic acid sequence or a fragment thereof. Such inhibitors when contacted with a cell expressing *chfr* inhibit the expression of (or inactivate) Chfr in that cell. For example, an inhibitor of *chfr* expression or function includes an oligonucleotide molecule which is preferably in an antisense orientation with respect to the nucleic acid sequence of *chfr*. As used herein, the term "antisense oligonucleotide" means a nucleic acid polymer, at least a portion of which is complementary to a *chfr* nucleic acid. "Antisense" refers particularly to the nucleic acid sequence of the noncoding strand of a double stranded DNA molecule encoding a protein, or to a sequence which is substantially homologous to the non-coding strand. As defined herein, an antisense sequence is complementary to the sequence of a double stranded DNA molecule encoding a protein. It is not necessary that the antisense sequence be complementary solely to the coding portion of the coding strand of the DNA molecule. The antisense sequence may be complementary to regulatory sequences specified on the coding strand of a DNA molecule encoding a protein, which regulatory sequences control expression of the coding sequences.

The antisense oligonucleotides of the invention preferably comprise between about fourteen and about fifty nucleotides. More preferably, the antisense oligonucleotides comprise between about twelve and about thirty

nucleotides. Most preferably, the antisense oligonucleotides comprise between about sixteen and about twenty-one nucleotides. The antisense oligonucleotides of the invention include, but are not limited to, phosphorothioate oligonucleotides and other modifications of oligonucleotides. Methods for synthesizing oligonucleotides, phosphorothioate oligonucleotides, and otherwise modified oligonucleotides are well known in the art [U.S. Patent No. 5,034,506; Nielsen *et al.*, 1991, Science 254: 1497].

2 Polypeptide/protein inhibitors

In another embodiment, another inhibitor composition of the invention includes a ligand which binds to Chfr polypeptide. Such a ligand is desirably an antibody which binds to Chfr, thereby inhibiting the function thereof. The term "antibody," as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope on an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, high affinity polyclonal antibodies, monoclonal antibodies, synthetic antibodies, chimeric antibodies, recombinant antibodies and humanized antibodies. Such antibodies may originate from immunoglobulin classes IgG, IgM, IgA, IgD and IgE. Such antibodies may include a Fab, Fab' or F(ab')₂, or Fc antibody fragment thereof which binds Chfr. Still another useful ligand is a single chain Fv antibody fragment which binds Chfr.

Another useful ligand is a recombinant construct comprising a complementarity determining region of an antibody, a synthetic antibody or a chimeric antibody construct or a humanized antibody construct which shares sufficient CDRs to retain functionally equivalent binding characteristics of an antibody that binds Chfr. By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the

antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

The antibodies of this invention are generated by conventional means utilizing the isolated, recombinant or modified Chfr or fragments thereof as
5 antigens of this invention. For example, polyclonal antibodies are generated by conventionally stimulating the immune system of a selected animal or human with a Chfr antigen, allowing the immune system to produce natural antibodies thereto, and collecting these antibodies from the animal or human's blood or other biological fluid. Preferably a recombinant version of Chfr is used as an immunogen. Monoclonal
10 antibodies (MAbs) directed against Chfr are also generated conventionally. Hybridoma cell lines expressing desirable MAbs are generated by well-known conventional techniques, e.g. Kohler and Milstein and the many known modifications thereof. Similarly desirable high titer antibodies are generated by applying known recombinant techniques to the monoclonal or polyclonal antibodies developed to these
15 antigens [see, e.g., PCT Patent Application No. PCT/GB85/00392; British Patent Application Publication No. GB2188638A; Amit *et al.*, Science, 233:747-753 (1986); Queen *et al.*, Proc. Nat'l. Acad. Sci. USA, 86:10029-10033 (1989); PCT Patent Application No. PCT/WO9007861; and Riechmann *et al.*, Nature, 332:323-327 (1988); Huse *et al.*, Science, 246:1275-1281 (1988)].

20 Given the disclosure contained herein, one of skill in the art may generate ligands or antibodies directed against Chfr by resort to known techniques by manipulating the complementarity determining regions of animals or human antibodies to the antigen of this invention. See, e.g., E. Mark and Padlin, "Humanization of Monoclonal Antibodies", Chapter 4, The Handbook of Experimental Pharmacology,
25 Vol. 113, The Pharmacology of Monoclonal Antibodies, Springer-Verlag (June, 1994); Harlow *et al.*, 1999, Using Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow *et al.*, 1989, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Bird *et al.*, 1988, Science 242:423-426.

Alternatively, Chfr antigens are assembled as multi-antigenic complexes [see, e.g., European Patent Application 0339695, published November 2, 1989] and employed to elicit high titer antibodies capable of binding the Chfr. Further provided by the present invention are anti-idiotypic antibodies (Ab2) and anti-anti-idiotypic antibodies (Ab3). Ab2 are specific for the target to which anti-Chfr antibodies of the invention bind and Ab3 are similar to Chfr antibodies (Ab1) in their binding specificities and biological activities [see, e.g., M. Wettendorff *et al.*, "Modulation of anti-tumor immunity by anti-idiotypic antibodies." In Idiotypic Network and Diseases, ed. by J. Cerny and J. Hiernaux J, Am. Soc. Microbiol., Washington DC: pp. 203-229, (1990)]. These anti-idiotypic and anti-anti-idiotypic antibodies are produced using techniques well known to those of skill in the art. Such anti-idiotypic antibodies (Ab2) can bear the internal image of Chfr and are thus useful for the same purposes as Chfr.

In general, polyclonal antisera, monoclonal antibodies and other antibodies which bind to Chfr as the antigen (Ab1) are useful to identify epitopes of Chfr to separate Chfr and its analogs from contaminants in living tissue (e.g., in chromatographic columns and the like), and in general as research tools and as starting material essential for the development of other types of antibodies described above. Anti-idiotypic antibodies (Ab2) are useful for binding the same target and thus may be used in place of Chfr to induce useful ligands to Chfr. The Ab3 antibodies are useful for the same reason the Ab1 are useful. Other uses as research tools and as components for separation of Chfr from other contaminants, for example, are also contemplated for the above-described antibodies.

Other ligands may include small chemical compounds that are screened in the ubiquitin-ligase assay described below and that are found to inhibit this enzymatic activity or other activities of Chfr. Such Chfr ligands or inhibitors may be identified and developed by the drug screening methods discussed in detail below.

3. Inhibitors as diagnostic reagents and kits

For use in diagnostic assays and kits, the above-described inhibitors of the *chfr* gene and Chfr polypeptide are preferably associated with a detectable label which is capable, alone or in concert with other compositions or

compounds, of providing a detectable signal. Where more than one reagent sequence or Chfr inhibitor is employed in a diagnostic method, the labels are desirably interactive to produce a detectable signal. Most desirably, the label is detectable visually, e.g. colorimetrically. A variety of enzyme systems operate to reveal a colorimetric signal in an assay, e.g., glucose oxidase (which uses glucose as a substrate) releases peroxide as a product which in the presence of peroxidase and a hydrogen donor such as tetramethyl benzidine (TMB) produces an oxidized TMB that is seen as a blue color. Other examples include horseradish peroxidase (HRP) or alkaline phosphatase (AP), and hexokinase in conjunction with glucose-6-phosphate dehydrogenase which reacts with ATP, glucose, and NAD⁺ to yield, among other products, NADH that is detected as increased absorbance at 340 nm wavelength.

Other label systems that may be utilized in the methods of this invention are detectable by other means, e.g., colored latex microparticles [Bangs Laboratories, Indiana] in which a dye is embedded may be used in place of enzymes to form conjugates with the inhibitor sequences or ligands and provide a visual signal indicative of the presence of the resulting complex in applicable assays. Still other labels include fluorescent compounds, radioactive compounds or elements. Preferably, each reagent or ligand is associated with, or conjugated to a fluorescent detectable fluorochromes, e.g., fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), coriphosphine-O (CPO) or tandem dyes, PE-cyanin-5 (PC5), and PE-Texas Red (ECD). All of these fluorescent dyes are commercially available, and their uses known to the art.

Detectable labels for attachment to reagent sequences and antibodies useful in diagnostic assays of this invention may be easily selected from among numerous compositions known and readily available to one skilled in the art of diagnostic assays. The diagnostic reagents and ligands of this invention are not limited by the particular detectable label or label system employed.

Methods for coupling or associating the label with the reagent sequence or ligand are similarly conventional and known to those of skill in the art. Known methods of label attachment are described [see, for example, Handbook of

Fluorescent Probes and Research Chemicals, 6th Ed., R.P. Haugland, Molecular Probes, Inc., Eugene, OR, 1996; Pierce Catalog and Handbook, Life Science and Analytical Research Products, Pierce Chemical Company, Rockford, IL, 1994/1995]. Thus, selection of the label and coupling methods do not limit this invention.

5 For convenience, the conventional reagents for ELISA or other diagnostic assays according to this invention may be provided in the form of kits. Such kits are useful for determining the absence (e.g., inactivation) or presence of *chfr* gene or Chfr polypeptide in a cell, particularly a tumor cell. Thus, such a kit will be useful in conducting the diagnostic assays discussed below, e.g., in determining if a cell is
10 tumorigenic, in determining the status of treatment of a cancer, etc. Such a diagnostic kit contains a nucleotide reagent sequence (e.g., a *chfr* antisense sequence), or Chfr inhibitor (e.g., an antibody capable of binding Chfr) of this invention. Alternatively, such kits may contain a simple mixture of such inhibitors or means for preparing a simple mixture. The kits also include instructions for performing the assay, microtiter
15 plates to which the inhibitors or nucleic acid sequences of the invention have been pre-adsorbed, various diluents and buffers, labeled conjugates for the detection of specifically bound compositions and other signal-generating reagents, such as enzyme substrates, cofactors and chromogens. Other components may include indicator charts for colorimetric comparisons, disposable gloves, decontamination instructions,
20 applicator sticks or containers, and a sample preparator cup. Such kits provide a convenient, efficient way for a clinical laboratory to diagnose the tumorigenic potential of a mammalian cell according to this invention

 Still another variant of a diagnostic kit for detecting the tumorigenic potential of a cell contains the components necessary for a Chfr-mediated ubiquitin
25 protein ligase assay, such as the assay described below. Such components may include the human E1 ubiquitin activating enzyme and the human E2 ubiquitin-conjugating enzyme, ubiquitin, ATP, an anti-ubiquitin antibody, an immobilized agent capable of binding labeled Chfr, as well as reagents necessary for performing gel electrophoresis and immunoblotting. Similarly, the non-biologic materials necessary for performing
30 such an assay (as described above) may be included in this kit.

One of skill in the art may be expected to vary the components of these diagnostic kits in obvious ways based on the knowledge in the art coupled with this disclosure. Such varied components are considered to be encompassed in this embodiment of the invention.

5 4. Inhibitors as Therapeutic Compositions of this Invention

Alternatively, an above-described inhibitor of Chfr of this invention may be employed therapeutically, and as such, is encompassed in a pharmaceutical composition. Such a composition includes a Chfr inhibitor (nucleotide or polypeptide or protein, or a small chemical compound) and a pharmaceutically-
10 acceptable carrier. As used herein, the term "pharmaceutically-acceptable carrier" means a chemical composition with which an appropriate Chfr inhibitor may be combined and which, following the combination, can be used to administer the appropriate Chfr inhibitor to a mammal. For example, suitable carriers include saline, buffered saline, and the like. In addition to the appropriate Chfr inhibitor, such
15 pharmaceutical compositions may also contain other ingredients known to enhance and facilitate drug administration. Other possible formulations, such as nanoparticles, liposomes, resealed erythrocytes, and immunologically based systems may also be used to administer an appropriate Chfr inhibitor according to the methods of the invention.

Also, as noted herein, pharmaceutical compositions of this
20 invention may include a combination of compounds comprising a Chfr inhibitor and another chemotherapeutic agent, particularly an agent which disrupts microtubule function. Among such agents that disrupt microtubule function include nocodazole, taxol and colcemid. Other such agents known in the art, or that may be developed in the future should be useful in this context.

25 Pharmaceutical compositions that are useful in the methods of the invention may be administered systemically by conventional therapeutic routes, e.g., intravenously, intraperitoneally, orally, via the mucosa, intramuscularly, subcutaneously, transdermally, topically, etc. Formulations suitable for the selected route can include, among others, oral solid formulations, ophthalmic, suppository,
30 aerosol, topical or other similar formulations that may be designed using information

known to one of skill in the pharmaceutical formulations art. Selection of the formulations and routes are within the skill of the art, and are not a limitation of this invention.

5 **D. *Methods Using the Compositions of this Invention***

According to the present discovery, *chfr* is required for regulation of the transition of cells from prophase to metaphase. Thus, the absence of functional *chfr* in a cell, or the presence of insufficient Chfr in a cell has ramifications with respect to whether a cell will become a tumor cell. Methods of this invention involve the use
10 of the *chfr* nucleotide sequences, Chfr polypeptide sequences as well as the Chfr inhibitors in diagnostic and therapeutic protocols.

1 *Diagnostic Methods of the Invention*

Because cells which lack proper checkpoints in the cell cycle are more likely to develop into tumor cells, the invention includes methods of identifying a
15 cell which is likely to become a tumor cell using the above-described compositions. In one embodiment, a method of determining tumorigenic potential of a mammalian cell includes examining the cell for the presence of, or mutations in, the *chfr* nucleic acid sequence. The substantial absence of, or mutation in, a *chfr* nucleic acid sequence indicates that the cell is predisposed to tumorigenesis, particularly upon exposure to an
20 agent or environment that is capable of inducing mitotic stress in the cell.

The detection of a *chfr* gene in a cell may be assessed in any ordinary nucleic acid expression assay, including techniques such as, Northern blotting with a suitable nucleic acid probe, Southern blotting, polymerase chain reaction (PCR), reverse transcriptase-PCR, RNase protection assays and *in situ* hybridization and the
25 like. Such assays may readily be employed *in vitro* by exposing a sample of tissue to be examined for tumorigenic potential to an anti-sense oligonucleotide, PCR primer or other *chfr* inhibitor of this invention. See, for example, the protocol of Example 2 below. Such assay techniques are conventional and the protocols for these assays are found in standard texts, such as Sambrook *et al*, cited above.

Another embodiment of a nucleic acid assay for use in determining the tumorigenic potential of a cell includes the steps of examining the cell for mutations in the *chfr* gene. The presence of mutations in the gene indicates that the cell is predisposed to tumorigenesis upon exposure to mitotic stress. This method involves isolating nucleic acid from the cells of selected species of mammal (preferably human) or other animal. This can be accomplished using either RNA or genomic DNA and using fragments of the *chfr* gene of this invention as the primers. The sequences obtained from the cells using RT-PCR for RNA or PCR for DNA are then amplified and the resulting gene sequenced to uncover any mutations. In order to examine the sequence for mutations, any conventional technique may be used, such as *in situ* hybridization. By this means the sequence from the cell under examination is compared to the sequence of a normal *chfr* gene to determine if the *chfr* gene of the cell bears a mutation. Techniques for comparison include conformation sensitive gel electrophoresis or single strand polymorphism analysis, among others. [See, Sambrook et al, or other conventional texts]. If desired, the sequence may be used to express a polypeptide, and that polypeptide may be tested to determine if it retains a function of Chfr, such as Chfr-mediated ubiquitin-protein ligase activity, or other functions as disclosed herein. Any mutations in these sequences that inactivate the Chfr function may be employed in methods and compositions of this invention.

In another embodiment, the invention provides a method of determining tumorigenic potential of a cell comprising examining the cell for the presence of Chfr polypeptide expression. The absence of a detectable level of Chfr polypeptide indicates that the cell is predisposed to tumorigenesis upon exposure to mitotic stress. The method also comprises determining whether or not Chfr is expressed at a lower than normal level in a cell, wherein a lower level of expression of Chfr in the cell, compared with expression of Chfr in an otherwise identical normal cell, is an indication that the cell will develop into a tumor cell.

Cells may be examined for expression of Chfr polypeptide using conventional protein and immunological assays, such as, without limitation, western immunoblotting with a suitable antibody, ELISA, immunofluorescence and

immunochemistry [see, e.g., Sambrook et al, and other texts for such assay steps]. Such assays may readily be employed *in vitro* by exposing a sample of tissue to be examined for tumorigenic potential to a Chfr inhibitor, e.g., an antibody of this invention as described above.

5 Still another embodiment of a method for determining the tumorigenic potential of a cell involves examining the cell for Chfr-mediated ubiquitin-protein ligase activity. As one embodiment, a diagnostic *in vitro* assay format involves capturing Chfr from cells on beads using antibodies that recognize Chfr. The beads may be conventional styrene or other beads which are conjugated to protein G or
10 protein A, which have the capability of capture antibodies, such as the anti-Chfr antibody. The beads are then incubated with the E1 and E2 ubiquitin enzymes, ubiquitin and ATP. In the presence of these enzymes, any Chfr protein normally produced in the cell will be ubiquitinated (will associate with ubiquitin). The beads are washed to remove all protein except the Chfr which is captured on the beads by the
15 protein A or protein G. Chfr is then eluted from the beads using, e.g., a sodium dodecyl-sulfate (SDS)-sample buffer. The released Chfr is then subjected to SDS gel electrophoresis and immunoblotting with an anti-ubiquitin antibody. If Chfr is ubiquitinated, then the anti-ubiquitin antibodies will recognize the Chfr protein indicating the cell has Chfr-mediated ubiquitin-protein ligase activity. If the cell has
20 such activity, the cell contains functional Chfr. The absence (or substantial reduction) of such activity indicates that the cell does not have functional Chfr and is therefore predisposed to tumorigenesis upon exposure to mitotic stress. See, e.g., Example 5 below. In this assay, the Chfr antibody released from the protein A or protein G conjugated beads may also be ubiquitinated and may also serve as a ubiquitination
25 substrate to monitor Chfr-mediated ubiquitin protein ligase activity in other formats of this assay.

As stated herein, cells which lack *chfr* function are more sensitive to agents which disrupt microtubule function than are cells which have *chfr* function. Thus, the invention further includes a method of determining the sensitivity of
30 a tumor cell in a mammal to agents which disrupt microtubule function or to other

chemotherapeutic agents. The methods described in detail above can be used to assess the cell for one or more of the characteristics including the substantial absence of a *chfr* gene; the substantial absence of Chfr protein; the substantial absence of Chfr-mediated ubiquitin-protein ligase activity; and/or a mutation in the *chfr* gene. The identification of any of these characteristics provides an indication that said tumor cell is sensitive to an agent which disrupts microtubule function. Thus, for example, the method can include assessing *ex vivo* the level of Chfr expression at the nucleic acid or protein level in the mammalian cell, which has been identified as a tumor cell. This experimental level is then compared to the level of Chfr expression in a non-tumor cell of the mammal. A lower level of expression of Chfr or the absence of Chfr expression or function in the cell compared with the level of expression of Chfr in an otherwise identical mammalian non-tumor cell, is an indication that the cell is sensitive to agents which disrupt microtubule function. This method can include assessing the cell for *chfr* gene mutations, as described above. Further, this method can include assessing the cell for Chfr-mediated ubiquitin-protein ligase activity, as described above.

Knowledge of the sensitivity of a tumor cell in a mammal to an agent which disrupts microtubule function may be used to determine the type of chemotherapeutic agent which might be administered to the mammal to kill the tumor cell. For example, the cells so identified may thereafter be exposed to a battery of such microtubule disrupting agents and/or other chemotherapeutic agents to enable the selection of the agent most effective in killing the tumor cells in an *ex vivo* or *in vivo* therapeutic context.

Similarly, as described above for nucleic acid assays, amplified RNA or DNA from the cells of a variety of mammalian (or other animal) species may be examined and/or expressed and assayed to detect mutations that inactivate the function of Chfr.

2 Therapeutic Methods of this Invention

As the data presented in the following examples establish, inactivation of Chfr function or a lower level of expression thereof in human cancer has two effects. First, it predisposes the cell to aneuploidy, as cells that condense their

chromosomes without having separated their centrosomes have difficulty forming an intact mitotic spindle. Second, it increases the sensitivity of cancer cells to mitotic stress. Thus, cancer cells lacking Chfr function would be sensitive to agents, such as nocodazole and taxol, that disrupt microtubule function, as demonstrated experimentally with the DLD1-neo and DLD1-*chfr* cells in the examples below.

Thus, the present invention also provides a therapeutic method of retarding the growth of, or killing, tumor cells, by inhibiting expression of Chfr in cells which are tumor cells. Since the development of tumor cells occurs via a vast number of mechanisms, the tumor cells to be killed need not necessarily have arisen due to a lack of adequate expression of Chfr. Indeed, the method of killing tumor cells is likely to be more effective in cells in which Chfr is expressed, and which have developed into tumor cells via a Chfr-independent mechanism. In this instance, inhibition of Chfr expression results in a tumor cell which is more sensitive to mitotic stress and is therefore more sensitive to agents, such as nocodazole and taxol, that disrupt microtubule function.

Thus, in another embodiment a therapeutic method of the invention comprises administering to a mammalian tumor cell, preferably *in vivo*, an inhibitor of Chfr expression or biological activity, such as the reagent antisense sequences and/or the protein ligands, and/or small chemical compounds described above in a dosage which is suitable to retard or inhibit expression or function of Chfr in the cell. This inhibition results in enhanced sensitivity of the tumor cell to mitotic stress, and thereby enhances the sensitivity of the cell to an agent which disrupts microtubule function. Such a method is also useful for killing a tumor cell. Thus, an optional step in this therapeutic method is administering to the tumor cell, or to the mammal bearing the tumor cell an agent which disrupts microtubule function in a suitable dosage selected for therapy. The administration of this second reagent may occur simultaneously with the Chfr inhibitor composition, or the administration of the agent which disrupts microtubule function may occur at some time after the Chfr inhibitor has produced its effect on the tumor cells. This method is useful in some embodiments in killing the cancer cell.

This method may be performed by administering the pharmaceutical compositions described above via any suitable therapeutic route, and selection of such route is not a limitation of this invention. Similarly the appropriate dosage of such pharmaceutical compositions may be determined by a physician, based on typical characteristics such as the physical condition of the patient, the disease being treated, the use of other therapeutic compositions, etc. In one embodiment, the pharmaceutical compositions useful for practicing the therapeutic methods of the invention may be administered to deliver a dose of between 1 ng/kg/day and 100 mg/kg/day. The dosages of the agent which disrupts microtubule function, such as taxol, are known to those of skill in the art. This invention is therefore not limited by the dosage selection, which is within the skill of the art.

E. Drug Screening and Screening for Chfr Inhibitors

The *chfr* nucleic acid sequences and Chfr polypeptides of this invention may also be used in the screening and development of chemical compounds, proteins or other compounds which have utility as therapeutic drugs for the treatment or diagnosis of cancer. Suitable assay methods for screening such potential drug compounds may be readily determined by one of skill in the art.

However, in one embodiment a method for identifying an inhibitor of *chfr* expression involves adding a test compound to a cell which is known to express Chfr at a specified level. The cell in which Chfr is expressed may be any cell found to express the *chfr* gene. Alternatively the cell may be one in which *chfr* is not normally expressed, but into which *chfr* has been introduced, by way of, for example, a plasmid or other vector, thereby enabling the expression of Chfr within the cell. After sufficient exposure to the test compound, the level of expression of *chfr* mRNA or protein is assessed according to the assays described in the examples below. This experimental level is then compared with the level of expression of *chfr* nucleic acid or Chfr protein in an otherwise identical cell to which the test compound has not been added. A lower level of expression of *chfr* nucleic acid or protein in a cell to which the test compound has been added, compared with the level in a cell to which the test compound has not

been added, is an indication that the test compound is capable of inhibiting Chfr expression.

Inhibitors of Chfr activity may also be screened by resort to assays and techniques useful in identifying drugs capable of binding to or interacting with the Chfr polypeptide and thereby inhibiting its biological activity in a cell that expresses Chfr. For example, another method of identifying a Chfr inhibitor comprising the steps of screening a test compound in a Chfr-mediated ubiquitin-protein ligase assay, such as the *in vitro* assay described above and in Example 5 below and variants thereof. The substantial absence of, or reduction in, said ligase activity in the assay in the presence of said test compound indicates that said test compound inhibits Chfr function. In one embodiment, the Chfr-mediated ubiquitin-protein ligase *in vitro* assay may be performed to screen small chemical compounds as inhibitors. To develop or screen small chemical compounds that inhibit Chfr-mediated ubiquitin protein ligase activity, it is preferred to employ purified, recombinantly-produced labeled Chfr protein (e.g., glutathione S-transferase (GST)-Chfr), E1 and E2 enzymes. These proteins may be conventionally recombinantly produced in, e.g., bacterial cells, insect cells or any of the cells described above for recombinant production in section B above. This assay may be performed by contacting a mixture which normally demonstrates Chfr-mediated ubiquitin-protein ligase activity with a test compound; and assaying said mixture and test compound for said activity. This mixture can contain, among other things, a labeled Chfr protein, the E1 enzyme, the E2 enzyme, ubiquitin and ATP. The assay can include the further steps of separating said labeled Chfr protein from said mixture, and performing gel electrophoresis thereon. Immunoblotting said gel with an anti-ubiquitin antibody permits detection of ubiquitinated Chfr in the gel. Identification of the presence of ubiquitin on the Chfr protein by said antibody demonstrates Chfr-mediated ubiquitin-protein ligase activity. If the antibody cannot bind any ubiquitin in the gel, the cell has no functional Chfr. The performance of such an assay when the mixture is in the presence or, or absence of a test compound and the comparison of the results obtained identifies the test compound as a Chfr inhibitor. Similarly assays that measure the response of cells to mitotic stress, such as those described in Example 4

below may be used for screening of chemotherapeutic drugs according to this invention.

Other conventional drug screening techniques may be employed using the proteins, antibodies or polynucleotide sequences of this invention. As one example, a method for identifying compounds which specifically bind to a Chfr polypeptide of this invention can include simply the steps of contacting a selected cell expressing Chfr with a test compound to permit binding of the test compound to Chfr and determining the amount of test compound, if any, which is bound to the Chfr. Such a method may involve the incubation of the test compound and the Chfr polypeptide immobilized on a solid support. Typically, the surface containing the immobilized ligand is permitted to come into contact with a solution containing the protein and binding is measured using an appropriate detection system. Suitable detection systems include those described above for diagnostic use.

Thus, through use of such methods, the present invention is anticipated to provide compounds capable of interacting with Chfr or the *chfr* gene or portions thereof, and either enhancing or decreasing Chfr's biological activity, as desired. Such compounds are believed to be encompassed by this invention.

Still other methods of drug screening for novel compounds that inhibit *chfr* expression at the nucleic acid or protein level involve computational evaluation and design. According to these methods, the three dimensional structure of the *chfr* gene and/or the polypeptide is determined and chemical entities or fragments are screened and selected for their ability to associate with the three dimensional structures. Suitable software for such analysis include docking software such as Quanta and Sybyl, molecular dynamics and mechanics programs, such as CHARMM and AMBER, the GRID program available from Oxford University, Oxford, UK. [P. J. Goodford, "A Computational Procedure for Determining Energetically Favorable Binding Sites on Biologically Important Macromolecules", *J. Med. Chem.*, 28 849-857 (1985)]; the MCSS program available from Molecular Simulations, Burlington, MA [A. Miranker and M. Karplus, "Functionality Maps of Binding Sites: A Multiple Copy Simultaneous Search Method", *Proteins: Structure, Function and Genetics*,

11:29-34 (1991)]; the AUTODOCK program available from Scripps Research Institute, La Jolla, CA [D. S. Goodsell and A. J. Olsen, "*Automated Docking of Substrates to Proteins by Simulated Annealing*", Proteins: Structure, Function, and Genetics, **8**:195-202 (1990)]; and the DOCK program available from University of California, San Francisco, CA [I. D. Kuntz *et al*, "*A Geometric Approach to Macromolecule-Ligand Interactions*", J. Mol. Biol., **161**:269-288 (1982)]. Additional commercially available computer databases for small molecular compounds include Cambridge Structural Database, Fine Chemical Database, and CONCORD database [for a review see Rusinko, A., Chem. Des. Auto. News, **8**:44-47 (1993)].

Once suitable chemical entities or fragments have been selected, they can be assembled into a single compound or Chfr inhibitor. Assembly may proceed by visual inspection of the relationship of the fragments to each other on the three-dimensional image displayed on a computer screen in relation to the 3D structure of Chfr. This would be followed by manual model building using software such as Quanta or Sybyl software, CAVEAT program [P. A. Bartlett *et al*, "CAVEAT: A Program to Facilitate the Structure-Derived Design of Biologically Active Molecules", in Molecular Recognition in Chemical and Biological Problems", Special Pub., Royal Chem. Soc. **78**, pp. 182-196 (1989)], which is available from the University of California, Berkeley, CA; 3D Database systems such as MACCS-3D database (MDL Information Systems, San Leandro, CA) [see, e.g., Y. C. Martin, "3D Database Searching in Drug Design", J. Med. Chem., **35**:2145-2154 (1992)]; and the HOOK program, available from Molecular Simulations, Burlington, MA.

Other molecular modeling techniques may also be employed in accordance with this invention. See, e.g., N. C. Cohen *et al*, "Molecular Modeling Software and Methods for Medicinal Chemistry", J. Med. Chem., **33**:883-894 (1990). See also, M. A. Navia and M. A. Murcko, "The Use of Structural Information in Drug Design", Current Opinions in Structural Biology, **2**:202-210 (1992). For example, where the structures of test compounds are known, a model of the test compound may be superimposed over the model of the structure of the invention. Numerous methods and techniques are known in the art for performing this step, any of which may be

used. See, e.g., P.S. Farmer, Drug Design, Ariens, E.J., ed., Vol. 10, pp 119-143 (Academic Press, New York, 1980); U.S. Patent No. 5,331,573; U.S. Patent No. 5,500,807; C. Verlinde, Structure, 2:577-587 (1994); and I. D. Kuntz, Science, 257:1078-1082 (1992). The model building techniques and computer evaluation systems described herein are not a limitation on the present invention.

Thus, using these computer evaluation systems, a large number of compounds may be quickly and easily examined and expensive and lengthy biochemical testing avoided. Moreover, the need for actual synthesis of many compounds is effectively eliminated. Once identified by the modeling techniques, the Chfr inhibitor may be tested for bioactivity using the assays described herein.

The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

EXAMPLE 1: IDENTIFICATION AND SEQUENCING OF *chfr*

To identify novel mitotic checkpoint genes, the Expressed Sequence Tag database was searched for cDNAs with FHA motifs. One of the positively identified cDNAs corresponded to EST clones #650972 and #1071323 and was sequenced in its entirety. See, e.g., Figs. 4A-4D. The cDNA [SEQ ID NO: 1] encodes a 664 amino acid protein [SEQ ID NO: 2] that contains within its N-terminus FHA and ring finger domains [Lovering *et al.*, 1993, Proc. Natl Acad. Sci. USA, 90:2112-2116; Borden *et al.*, 1995, EMBO J. 114:1532-1541; Hofmann, K. & Bucher, P Trends Bioch. Sci. 20, 347-349 (1995)]. Within its C-terminus is found a cysteine-rich region that is highly conserved between human and mouse, but which does not display significant similarity to any protein in the GenBank database, including the recognized zinc-binding domains (Fig. 1A). As described below, this protein functions as a mitotic cell cycle checkpoint. It is referred to herein as Chfr (CHeckpoint with FHA and Ring finger). Chfr may be a member of a small family of proteins that contain FHA and Ring Finger

domains. Other members of this small family are Dmal (Defective in mitotic arrest 1), an *S. pombe* mitotic checkpoint protein [Murone and Simanis, 1996, EMBO J., 15:6605-6616], and Yhr 115c and Ynl16w, the predicted protein products of two, as yet uncharacterized, *S. cerevisiae* open reading frames (Fig. 1A). Dmal, Yhl15c and Ynl16w are highly related to each other, whereas Chfr bears less similarity to these three proteins and may not be, therefore, their human ortholog (Figs. 1A to 1C).

The FHA domain of Chfr has highest similarity to the FHA domain of Rad53/Spkl (Fig. 1B), a DNA damage checkpoint protein kinase [Stem *et al.*, 1991, Mol. Cell. Biol. 11:987-1001; Allen *et al.*, 1994, Genes Dev. 8:2401-2415], whereas the ring finger is most similar to the ring finger of the *Varicella zoster* virus transactivator ICP0 (Fig. 1C). Apart from its role as a transactivator [Moriuchi *et al.*, 1992, J. Virol., 66:7303-7308], ICP0 interacts with the kinetochore and interferes with progress through mitosis. These two activities require an intact ring finger [Everett *et al.*, 1999, EMBO J., 18:1526-1538].

No proteins with significant similarity to the C-terminus of Chfr were identified.

EXAMPLE 2: METHODS AND MATERIALS EMPLOYED IN THE FOLLOWING EXPERIMENTS

The materials and methods used in the experiments presented herein are now described.

A. Chfr expression in normal tissues and cancer cell lines

Chfr expression was examined at the mRNA and protein levels. For analysis at the mRNA level, a *chfr* probe corresponding to the Eco47III fragment of EST clone # 650972 was prepared by ³²P-labeling (Oligolabeling Kit, Pharmacia, Piscataway, NJ) and was hybridized with a human multiple tissue Northern blot (Clontech Inc., Palo Alto, CA) and Northern blots prepared with mRNA isolated from cancer cells lines using the Quickprep Micro mRNA Purification Kit (Pharmacia, Piscataway, NJ). The extent of hybridization was monitored by autoradiography.

For analysis of expression of chfr protein, cells were recovered from tissue culture plates with trypsin, pelleted and lysed in cell lysis (CL) buffer (50 mM Tris, pH 8, 120 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 μ M staurosporine, 15 mM NaF, 1 mM sodium vanadate, 1 μ g/ml aprotinin and 1 μ g/ml leupeptin). The proteins in the whole cell lysates were resolved by denaturing gel electrophoresis and transferred to PVDF membranes. Immunoblotted Chfr protein was detected using an affinity-purified rabbit polyclonal antibody prepared using purified recombinant histidine-tagged Chfr protein as the antigen (Research Genetics).

B. Cell Culture

All cancer cells were grown in DMEM supplemented with glutamine, penicillin, streptomycin and 10% fetal bovine serum (Life Sciences). Normal human epidermal keratinocytes and osteoblasts were grown in KGM2 and OGM media, respectively (Clonetics). The cells were examined either non-synchronized or synchronized. For synchronization, the cells were treated with 2 mM thymidine for 16 hours, then with 0.25 mM thymidine/deoxycytidine for 9 hours, and then with 0.5 μ g/ml aphidicolin for 20-24 hours. The cells were washed three times with PBS between each step [Janss *et al.*, 1998, *Exp. Cell Res.*, 243:29-38]. To induce mitotic stress, synchronized or non-synchronized cells were exposed to 0.5 μ g/ml nocodazole, 5 μ M taxol or 0.5 μ g/ml colcemid.

C. Ectopic Chfr Expression

The mammalian expression plasmid, pSV2-HA*chfr*, which directs expression of chfr in mammalian cells, was constructed from pSV2hp53BS by replacing the p53 insert with an insert encoding full-length Chfr protein fused at its N-terminus to an HA tag [Wieczorek *et al.*, 1996, *Nature Med.*, 2:1143-1146]. pSV2-HA*chfr*V₅₈₀M (also pSV2-HA*chfr*M₅₈₀), which was derived from pSV2-HA*chfr* by site-directed mutagenesis, encodes a chfr protein bearing a substitution of Val₅₈₀ with Met. pSV2-HA*chfr*- Δ FHA was derived from pSV2-HA*chfr* by site-directed mutagenesis and lacks nucleotide residues 2-142 of *chfr* of Figs. 4A-4D [SEQ ID NO. 1].

Stable transfectants were prepared by transfecting DLD1 or U20S cells with 5 µg pSV2-HAchfr or pSV2-HAchfr-M₅₈₀, or 5 µg pSV2 vector without insert and 1 µg pSV7neo plasmid [Wieczorek *et al.*, supra] using Fugene-6 transfection reagent (Roche). Stably transfected cells were selected with G418. For transient expression, DLD1 or U20S cells were transfected with 5 µg pSV2-HAchfr or pSV2-HAchfrM₅₈₀ or pSV2 plasmid without insert and 2 µg of a plasmid expressing green fluorescent protein using Fugene-6. SAOS2 cells were transfected as described above, except that the plasmids expressing Chfr were cotransfected with a plasmid expressing green fluorescent protein (GFP).

D. Mitotic index and centrosome staining

Cells were grown on 8-well culture slides coated with human fibronectin (Becton Dickinson) and were examined as either non-synchronized or synchronized cells. The cells were prepared for microscopy by washing them three times with KM buffer (10 mM MES, pH 6.2, 10 mM NaCl, 1.5 mM MgCl₂, 2.5% glycerol), fixing with 1% paraformaldehyde in 0.5 X KM buffer for 15 minutes, washing once with 0.2% Triton X-100 in phosphate buffer saline (PBS) for 20 minutes and three times with PBS. For centrosome staining, the cells were incubated for 1 hour with a 1:500 dilution of autoimmune serum Ab598 in PBS, washed three times with PBS, incubated with a Texas Red-conjugated anti-human secondary antibody (Vector Labs) diluted 1:200 in PBS and washed again three times with PBS. For DNA staining, the cells were incubated with DAPI (2 mg/ml in PBS). The slides were sealed with coverslips using Fluoromount-G (Upstate Biotechnology) and visualized with a fluorescence microscope (Leica). Separate images, acquired using filters corresponding to the excitation maxima of DAPI and Texas Red, were merged with IRIX image tools (Silicon Graphics).

E. Determination of viability in response to mitotic stress

Cells synchronized by a sequential thymidine-aphidicolin block were either not exposed to mitotic stress or exposed to 0.5 µg/ml nocodazole or taxol for a 4 hour period starting 12 hours after aphidicolin release or release from the G1-S block. The short term response to mitotic stress was evaluated by examining the cell

cycle profile at the time of nocodazole removal, 24 or 48 hours later. At the indicated time points, the cells were recovered from the tissue culture plates with trypsin, fixed in 70% ethanol for 10 minutes and incubated with propidium iodide and DNase-free RNase (Roche) in PBS containing 1% fetal bovine serum and 2% Tween-20. The nuclear morphology of the cells was visualized by fluorescence microscopy. The DNA content of the cell population was determined by flow cytometry.

To evaluate the long term response of the cells to survive exposure to mitotic stress, at the time of nocodazole removal, the cells were replated at a density of 200 cells per 100 mm diameter tissue culture dish. The cells were allowed to replicate and colonies were counted 3 weeks later.

F. *Cdc2 kinase activity*

Whole cell extracts, prepared as described above, were incubated with anti-cyclin B antibody (Santa Cruz) coupled to protein G beads (Pharmacia) in CL buffer for 1 hour. The beads were washed three times with CL buffer and then twice with cdc2 kinase (CK) buffer (50 mM HEPES, pH 7.0, 10 mM MgCl₂, 10 mM MnCl₂, 200 mM NaCl). The beads were then incubated with 1 µg histone H1 (Upstate Biotechnology) in CK buffer supplemented with 30 mM DTT, 0.06 µM ATP and 1 µCi ³²P-γ-ATP for 20 minutes at 30°C, at which time the reactions were subjected to denaturing gel electrophoresis. Phosphorylation of histone H1 was detected by autoradiography.

EXAMPLE 3: DETECTION OF CHFR MUTATIONS IN CANCER CELL LINES

The following experiment was an examination of whether the *chfr* gene is mutated in any of the cancer cell lines (including SW480, DLD1, HT29, HCT116, SAOS2, U2OS, IMR5 and NGP), e.g., those that express mRNA and protein or those that do not. Specifically examined was whether a mutation in *chfr* gene in a cancer cell line leads to synthesis of a functionally inactive protein. For this purpose, mRNA was isolated from these cancer cell lines (Quickprep Micro mRNA Purification Kit, Pharmacia) and was used as template for first-strand cDNA synthesis (Retroscript, Ambion). Synthetic oligonucleotides were used to amplify regions of the *chfr* cDNA

by RT-polymerase chain reaction (Platinum Taq, Life Sciences or GC-rich PCR system, Roche). The amplified regions spanning the entire Chfr coding sequence were sequenced using four-color fluorescent dideoxy terminators (Big Dyes, Perkin Elmer). The oligonucleotides used to generate the PCR fragment served as sequencing primers. Specific primer pairs used to amplify regions of the *chfr* cDNA by PCR are reported in Table 1.

TABLE 1

5' Primer	SEQ ID NO	3' Primer	SEQ ID NO	Region amplified [nts of SEQ ID NO: 1]
TGTCTCTTGACAGCGG C	9	CATGGAACACATTTTCCTT G	10	66-562
AAAGAATTCTGGAAGA TACCAGCACCAG	11	AAAAAGCTTGGCAGATGAT GCATGTCAG	12	352-1055
AAAGAATTCCTCCCT AAAGGAAGTG	13	AAAAAGCTTTCAACGTCTG ACAGCTC	14	771-1376
AAGAAAATGAGAGGA GATGG	15	GGTTGAGCTCACAAAACG	16	904-1753
AAGAAAATGAGAGGA GATGG	17	TCCAGACACTTGTCACC	18	904-1772
AAGAAAATGAGAGGA GATGG	19	AGACAGCAGAAACACTCC	20	904-1902
ACCACATCCTCAACAA CC	21	GGTTGAGCTCACAAAACG	22	1187-1753
ACCACATCCTCAACAA CC	23	TCCAGACACTTGTCACC	24	1187-1772
ATACCTCATCCAGCAT CC	25	GGTTGAGCTCACAAAACG	26	1215-1753
ATACCTCATCCAGCAT CC	27	TCCAGACACTTGTCACC	28	1215-1772
ATACCTCATCCAGCAT CC	29	AGACAGCAGAAACACTCC	30	1214-1902
AAAGAATTCCAGCCTT TCTGCCACC	31	AAAAAGCTTTCCACAGAAG AGTCACCC	32	1625-2279

Analysis of the sequences indicated that U2OS was the only cell line that displayed a sequence variation (see, Figs. 3A and 3B). This experiment detected the presence of a C→T transition in the non-coding strand in Chfr cDNA prepared from U2OS cells, leading to substitution of Val₅₈₀ with Met₅₈₀ in the highly conserved C-terminal cysteine-rich region of Chfr and affecting the entire pool of U2OS mRNA. The sequencing did not reveal any wildtype sequence. The transition involves substitution of a CG dinucleotide (which is a mutagenesis hot-spot [Holliday, R. & Grigg, G.W., 1993, Mutat. Res., 285, 61-67]) in the non-coding strand with a TG dinucleotide and is typical of mutations that occur when methylated cytosines undergo deamination to form thymine [You *et al.*, 1998, Mutation Res., 420:55-65]. Furthermore, the substitution targets a region of Chfr protein that is very highly conserved in evolution and was not detected in SW480 cells, which retain wild-type Chfr function.

EXAMPLE 4: EXPERIMENTS AND RESULTS

Using the methods outlined in Example 2 above, the following data was collected and interpreted involving *chfr* and its biological function in regulating the response of cells to mitotic stress. As determined below, the correlation between Chfr expression and mitotic index in response to nocodazole is consistent with a role of Chfr as a cell cycle checkpoint.

In normal human tissues, expression at the mRNA level was determined by Northern blotting. In the resulting gels, Chfr expression was found in normal tissue of the heart, brain, placenta, lung, liver, muscle, kidney and pancreas. Thus, Chfr expression was ubiquitous in normal human tissues, providing evidence that its function is not tissue-specific.

Chfr expression was further examined in a panel of eight human cancer cell lines, including SW480, DLD1, H129, HCT116, SAOS2, U2OS, IMR5 and NGP. At the mRNA level, three of the eight cell lines did not express detectable *chfr* (DLD1, HCT116 and IMR5). Expression at the protein level was also determined by Western immunoblotting with an affinity-purified polyclonal antibody raised against

recombinant Chfr protein. The cell lines DLD1, HCT116 and IMR5 did not express Chfr protein. The molecular basis for the lack of Chfr expression does not involve deletion of both copies of the *chfr* gene, since by Southern blotting all eight of the above-mentioned cancer cell lines have at least one copy of the *chfr* gene.

5 Nevertheless, the high frequency of undetectable Chfr expression prompted an examination of whether *chfr* is mutated in these cancer cell lines, including those that express mRNA and protein, as described in Example 3 above.

Because Chfr and Dmal share structural domains, the possibility that *chfr* is a mitotic checkpoint gene was examined. The eight cancer cell lines described above
10 were treated with nocodazole, which induces mitotic stress by depolymerizing the microtubules that form the mitotic spindle. The ability of cells to undergo mitotic arrest was examined by staining the cells with DAPI 16 hours later. The cells were scored for Mitotic Index. Mitotic index is the fraction of cells that had condensed
15 chromosomes, and represents cells that are in metaphase or anaphase. For the cell lines that had no detectable Chfr expression and for the U20S cells, which expressed the variant *chfr* gene, the fraction of cells that had condensed chromosomes (mitotic index) was high, indicating arrest in metaphase. In contrast, the mitotic index of the cell lines that expressed wild-type Chfr was low (Fig. 2), which indicates either that
20 these cells were not arrested in the cell cycle or that they were arrested in some phase of the cell cycle other than metaphase or anaphase.

To determine whether Chfr accounted for the different response, DLD1 and U20S cells were prepared to stably express HA-tagged wild-type Chfr or Chfr with the Val₅₈₀ to Met substitution (M₅₈₀) or just the neo selectable marker. The cells expressing
25 neo or Chfr-M₅₈₀ had a high mitotic index in response to nocodazole, like the parent cells. However, the cells expressing wild-type Chfr had a low mitotic index (Fig. 7A). Near normal levels of wild-type Chfr protein were sufficient to affect the response to mitotic stress, since the ectopic Chfr protein in the stably-transfected DLD1-*chfr* cells was expressed at levels similar to those of endogenous Chfr in primary human cells (NHEK, NHOST and NHF; obtained from Clonetics). Furthermore, the different
30 effects of wild-type Chfr and Chfr-M₅₈₀ could not be attributed to differences in protein

expression, as determined by immunoblotting. Similar results were obtained with transiently transfected U2OS and DLD1 cells (Fig. 3C). Thus the nucleotide transition targeting *chfr* in U2OS cells is a mutation, because it inactivates the function of Chfr.

To further strengthen the link between Chfr and the response to mitotic stress, experiments were performed to determine whether a dominant negative Chfr mutant would alter the behavior of cells, such as SAOS2, that express wild-type Chfr and have a low mitotic index in response to mitotic stress. Chfr- Δ FHA, a Chfr protein with deletion of residues 2-142 encompassing the FHA domain, was identified as a dominant negative mutant by studying its function in DLD 1 cells. Its effect on the response of SAOS2 cells to mitotic stress was studied by transiently-transfecting these cells with plasmids that express Chfr- Δ FHA or wild-type Chfr or no Chfr protein, together with a plasmid expressing GFP, as a marker. 36 hours later, mitotic stress was induced by exposure to taxol and the mitotic index was determined 8 to 16 hours later. Protein levels were determined by immunoblotting with an antibody that recognizes the N-terminal HA tag of the expressed Chfr proteins.

About 50% of the cells expressed GFP, but the variable level of expression made it difficult to define a threshold above which a cell would be considered GFP-positive. Thus, to avoid any bias, the mitotic index was calculated for the entire cell population. Expression of wild-type Chfr had no effect as compared to cells transfected with empty vector (Fig. 7B). However, Chfr- Δ FHA, whose level of expression was equivalent to that of wild-type Chfr led to a five-fold increase in the mitotic index at the 12, 14 and 16 hour timepoints, indicating a checkpoint defect. At the 8 hour timepoint, the mitotic index was low, similar to cells that lack Chfr (e.g. DLD 1 and HCT 116), which begin to show a high mitotic index in response to mitotic stress 12-16 hours after addition of nocodazole or taxol. The effect of Chfr- Δ FHA in this assay was through dominant inhibition of endogenous wild-type Chfr based on an analysis of its function in transiently-transfected DLD1 cells, which lack endogenous Chfr. Chfr- Δ FHA had no effect on the mitotic index of DLD 1 cells exposed to mitotic stress, as compared to vector control, but inhibited the ability of wild-type Chfr to

decrease the mitotic index. In the same assay, Chfr-M₃₈₀ did not act as a dominant negative mutant (Fig. 7C).

The low mitotic index of nocodazole-treated cells expressing wild-type Chfr could indicate either cell cycle arrest at some point in the cell cycle before entry into metaphase or due to exit from mitosis due to failure to arrest in metaphase. To distinguish between these possibilities, the effect of Chfr expression in synchronized cells was examined. The stably-transfected DLD1-chfr and DLD1-neo cells described above were synchronized by consecutive thymidine and aphidicolin blocks at the G1-S boundary. Aphidicolin was then washed off. These cells were allowed to proceed through the cell cycle (mitosis) either in the presence or absence of mitotic stress (i.e., the cells were either treated with nocodazole 12 hours after release from cell cycle arrest or not exposed to nocodazole). Progression through the cell cycle was monitored by measuring the mitotic index and by flow cytometric analysis of the DNA content of the cells.

In the absence of nocodazole (i.e., mitotic stress), Chfr had no effect on cell cycle progression, including entry and exit from mitosis, as determined by analysis of the mitotic index and cdc2 kinase activity performed as described in Example 2, and measured as a function of time after release from aphidicolin-induced cell cycle arrest. However, Chfr delays entry into metaphase in response to mitotic stress (Figs. 8A and 8B).

Similar results were obtained when mitotic stress was induced by colcemid or taxol, two other drugs that affect microtubule dynamics (Figs. 8A and 8B). Thus, Chfr regulates the prophase to metaphase transition in response to mitotic stress. Consistent with this role, the timing of induction of mitotic stress was critical for Chfr to delay entry in metaphase. Chfr did not affect cell cycle progression when nocodazole was added as the cells were entering metaphase 14 hours after aphidicolin release. In this case, both DLD1-neo and DLD1-chfr cells arrested in metaphase or entered metaphase with the same kinetics, as in the absence of mitotic stress (see, e.g., Figs. 8A and 8B). Essentially identical results were obtained when U2OS cells stably-transfected with plasmids expressing neo or wild-type chfr were examined.

Furthermore, human primary epidermal keratinocytes and osteoblasts also exhibited a delay in metaphase entry in response to mitotic stress (Figs. 8C and 8D).

To correlate the timing of the Chfr effect with progress through the cell cycle, synchronized cells were stained with DAPI to monitor chromosome condensation and with an antibody that recognizes the centrosomes. A series of images was generated depicting disjunction of chromosome condensation and centrosome separation in cells lacking Chfr. Representative views of DLD1-neo and DLD1-chfr cells at 12, 14 and 16 hours after release from aphidicolin arrest were generated. DNA was stained with DAPI and centrosomes were identified by immunofluorescence. At 12 hours after aphidicolin release, the nucleus exhibited no signs of chromosome condensation and the centrosomes, which duplicate in S phase, were physically next to each other, suggesting that the cells were in G2. At 14 hours, most of the cells were in prophase since the centrosomes had separated from each other, while the chromosomes had not yet condensed. At 16 hours, most of the cells were in metaphase with condensed chromosomes between the separated centrosomes.

Representative views of DLD1-neo and DLD1-chfr cells at the 14 hour time point after release from aphidicolin arrest were also generated. These cells were exposed to nocodazole 12 hours after release from cell cycle arrest. When nocodazole was added 12 hours after aphidicolin release, centrosome separation at the 14 hour time point was inhibited in both DLD1-chfr and DLD1-neo cells. At this time point, a significant number of DLD1-neo cells had condensed chromosomes despite failing to separate their centrosomes, whereas the DLD1-chfr cells typically did not exhibit chromosome condensation.

Cyclin B/cdc2 activity was high in synchronized DLD1-neo and DLD1-chfr cells treated with nocodazole 12 hours after release from the G1-S block. Persistence of high cyclin B/cdc2 activity indicates arrest in mitosis; DLD1-neo cells were arrested in metaphase due to activation of the spindle checkpoint, DLD1-chfr cells were arrested initially in prophase by the Chfr checkpoint and later in metaphase by the spindle checkpoint (Figs. 8A and 8B). The high cyclin B/cdc2 activity in cells whose entry into metaphase is delayed by Chfr distinguishes the Chfr checkpoint from the G2 DNA

damage checkpoint, which delays entry into mitosis by inhibiting cyclin B/cdc2 [Weinert, T. 1998 Cell 94:555-558].

The ability of Chfr to affect progression through the cell cycle only in the presence of mitotic stress provides evidence that Chfr is a mitotic checkpoint.

5 Furthermore, Chfr has an effect only when nocodazole was added prior to completion of prophase, which suggests that Chfr monitors events that occur during prophase. The nature of the event being monitored is likely centrosome separation. The disjunction of chromosome condensation from centrosome separation in the absence of the Chfr checkpoint is theorized to lead to aberrant chromosome segregation during anaphase and, consequently, to decreased cell viability.

10 To further support the hypothesis that Chfr is a mitotic checkpoint, Chfr was examined to determine whether it affects cell viability in response to mitotic stress. Stably-transfected DLD1-neo and DLD1-chfr cells were synchronized by sequential thymidine-aphidicolin blocks and exposed to 0.5 μ g/ml nocodazole or taxol for a 4
15 hour period starting 12 hours after aphidicolin release or release from the G1-S block. The short-term response of the cells to mitotic stress was evaluated by examining cellular DNA content by flow cytometry and their nuclear morphology under the fluorescent microscope 48 hours later and the cell cycle profile at the time of nocodazole removal, 24 or 48 hours later. For microscopic examination, the cells
20 were recovered from the tissue culture plates with trypsin, fixed in 70% ethanol for 10 minutes and incubated with propidium iodide and DNase-free RNase (Roche) in PBS containing 1% fetal bovine serum and 2% Tween-20. Further, after staining the cells with DAPI, the cells were inspected by fluorescence microscopy 64 hours after release from G1-S block.

25 The DLD1-chfr cells exhibited the normal DNA content profile of cycling cells and normal nuclear morphology. The DNA content profile of the DLD1-neo cells was also normal, but their nuclear morphology was clearly aberrant. About half of all cells with a 4N DNA content exhibited fragmented nuclei suggesting that they had not completed mitosis properly.

To the long term response of the cells to survive exposure to mitotic stress, synchronized DLD1-neo and DLD1-chfr cells were transiently exposed to nocodazole or taxol, as described above (e.g., exposed to nocodazole or taxol 12 hours after release from the G1-S block for a 4 hour period). At the time of nocodazole removal, the cells were replated at a density of 200 cells per 100 mm diameter tissue culture dish and then allowed to form colonies over a three-week period. DLD1-neo cells showed a decrease in the number of colony-forming units (CFUs) in response to mitotic stress, whereas for the DLD1-chfr cells the number of CFUs was unaffected by mitotic stress (Fig. 5). This provides additional evidence that Chfr expression leads to a low mitotic index in response to nocodazole.

The *chfr* gene molecularly defines the existence of a novel checkpoint that regulates entry into metaphase. The Chfr checkpoint was evident in primary human cells, but was inactivated in four out of eight examined human cancer cell lines. In the absence of the Chfr checkpoint, cells subjected to mitotic stress condensed their chromosomes despite failing to separate their centrosomes. It is presently theorized that Chfr monitors centrosome separation, rather than some other mitotic stress-sensitive event that occurs in prophase. The molecular mechanism by which Chfr delays cell cycle progression and the frequency of Chfr inactivation in primary tumors are being studied. Analysis of a small number of cancer cell lines raises the possibility that Chfr is inactivated more frequently than all known spindle checkpoint genes combined. If Chfr is inactivated in human cancer, then its inactivation may underlie the increased sensitivity of cancer cells to antimitotic drugs.

EXAMPLE 5: CHFR HAS UBIQUITIN-PROTEIN LIGASE ACTIVITY

Recombinant *E. coli* bacterial cells that have been genetically engineered to express the E1 ubiquitin-activating enzyme and the E2 ubiquitin-conjugating enzyme (either UbchD2 or Ubch8) and a fusion protein comprised of glutathione S-transferase fused to the N-terminus of Chfr were lysed. The lysates were incubated in the presence of ubiquitin and ATP and the reaction allowed to proceed for 20 minutes at 30°C. GST-Chfr was captured on glutathione beads, eluted with SDS sample buffer,

and resolved by SDS-PAGE. The SDS gel was immunoblotted with antibodies that recognize ubiquitin. Reactions were performed with full-length Chfr fused to GST and reactions were performed with Chfr lacking amino acid residues 1-280 of SEQ ID NO: 2, which contain the FHA domain. If the GST-Chfr is ubiquitinated, then the anti-ubiquitin antibodies will recognize the GST-Chfr protein, indicating that the GST-Chfr has ubiquitin-protein ligase activity.

Using this ubiquitin-protein ligase assay *in vitro*, efficient ubiquitination of GST-Chfr was detected (data not shown). As with other E3 ligases, ubiquitination required the presence of both E1 and E2 and demonstrated E2-specificity, since the E2 ubiquitin-conjugating enzyme UbchD2, supported the ubiquitin-protein ligase activity of Chfr, whereas another E2, Ubch8, did not function in this assay. The E3 ligase activity of Chfr was dependent on the integrity of its ring finger, since substitution of Cys₃₂₅ with Ala, abrogated ligase activity. In contrast, the FHA domain of Chfr was not required for ligase activity *in vitro*, since a GST-Chfr protein that lacks amino acid residues 1-280 of human Chfr [SEQ ID NO: 2], was active. Finally, GST by itself did not have ubiquitin-protein ligase activity in this assay.

These preliminary results were performed with crude bacterial lysates. However, all the recombinant proteins in these extracts could be visualized by Coomassie blue staining. The levels of expression of UbchD2 and Ubch8 were similar, as were the levels of all the GST-Chfr fusion proteins. Thus, the different activities observed with these different proteins were not simply due to differences in the levels of protein expression.

To determine whether ubiquitin-protein ligase activity is required for checkpoint function, the Chfr mutant that substitutes Cys₃₂₅ of the ring finger with Ala was stably-expressed in DLD1 cells. These cells were then exposed to mitotic stress and examined for entry into metaphase. The mitotic index of unsynchronized DLD1 cells exposed to nocodazole is high, indicating the absence of a checkpoint that would delay entry into metaphase in response to mitotic stress. Expression of wild-type Chfr restores the checkpoint leading to a low mitotic index. Expression of Chfr-A₃₂₅, which lacks ubiquitin-protein ligase activity, did not lead to a low mitotic index (see Fig. 6)

indicating that the checkpoint function of Chfr is dependent on its E3 ligase activity. Expression of Chfr-A₃₂₅ in the transfected DLD1 cells was monitored by immunoblotting and was shown to be equivalent to the expression of wild-type Chfr.

5 The *chfr* gene molecularly defines the existence of a novel checkpoint that regulates entry into metaphase. The Chfr checkpoint was evident in primary human cells; but was inactivated in four out of eight examined human cancer cell lines. In the absence of the Chfr checkpoint, cells subjected to mitotic stress condensed their chromosomes despite failing to separate their centrosomes. It is presently theorized that Chfr monitors centrosome separation, rather than some other mitotic stress-
10 sensitive event that occurs in prophase. The molecular mechanism by which Chfr delays cell cycle progression and the frequency of Chfr inactivation in primary tumors are being studied. So far, analysis of a small number of cancer cell lines raises the possibility that *chfr* is inactivated more frequently than all known spindle checkpoint genes combined. The inactivation of *chfr* in human cancer is theorized to underlie the
15 increased sensitivity of cancer cells to antimitotic drugs.

The disclosures of each and every patent, patent application, and publication cited herein, including that of provisional US patent application No. 60/146,194 are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other
20 embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid sequence of a mitotic checkpoint gene, *chfr*, which encodes a Chfr protein having a Forkhead-associated domain and a Ring Finger, wherein said protein is required for regulation of the transition of cells from prophase to metaphase.
2. The sequence according to claim 1, which is selected from the group consisting of:
 - (a) SEQ ID NO: 1 or an anti-sense sequence thereof,
 - (b) a sequence encoding at least amino acids 31 to 103 of SEQ ID NO: 2 or an anti-sense sequence thereof,
 - (c) a sequence encoding at least amino acids 303 to 346 of SEQ ID NO: 2 or an anti-sense sequence thereof,
 - (d) a sequence encoding at least amino acids 476 to 641 of SEQ ID NO: 2 or an anti-sense sequence thereof,
 - (e) a sequence encoding at least amino acids 31 to 103, amino acids 303 to 346 and 476 to 641 of SEQ ID NO: 2 or an anti-sense sequence thereof; and
 - (f) a sequence having a homology of at least 50% to the sequences (a) through (e) according to a selected algorithm and encoding a protein or peptide having ubiquitin-protein ligase activity.
3. The sequence according to claim 1, which is synthetically or recombinantly produced.
4. The sequence according to claim 1, which is associated with a detectable label.

5. The sequence according to claim 1, which is present as a wild-type gene in normal human epidermal keratinocytes and normal human osteoblasts,
6. The sequence according to claim 1 that encodes a polypeptide that delays entry of a human cell into metaphase in response to mitotic stress.
7. A substantially pure preparation of a polypeptide comprising a Forkhead-associated domain and a Ring Finger domain, wherein said protein is required for regulation of the transition of a normal human cell from prophase to metaphase.
8. The polypeptide according to claim 7, which is selected from the group consisting of
 - (a) SEQ ID NO: 2 or an complementary sequence thereof,
 - (b) a sequence comprising at least amino acids 31 to 103 of SEQ ID NO: 2 or an complementary sequence thereof;
 - (c) a sequence comprising at least amino acids 303 to 346 of SEQ ID NO: 2 or an complementary sequence thereof;
 - (d) a sequence comprising at least amino acids 476 to 641 of SEQ ID NO: 2 or an complementary sequence thereof;
 - (e) a sequence comprising at least amino acids 31 to 103, amino acids 303 to 346 and 476 to 641 of SEQ ID NO: 2 or an complementary sequence thereof; and
 - (f) a sequence having a homology of at least 50% to the sequences (a) through (e) according to a selected algorithm and comprising a protein or peptide having ubiquitin-protein ligase activity.
9. The polypeptide according to claim 7, which is expressed in normal human epidermal keratinocytes and normal human osteoblasts.

10. The polypeptide according to claim 7 that delays entry of a human cell into metaphase in response to mitotic stress.

11. A method of determining tumorigenic potential of a cell comprising examining said cell for the presence of *chfr* nucleic acid sequence in said cell, wherein the absence of said *chfr* nucleic acid sequence indicates that said cell is predisposed to tumorigenesis upon exposure to mitotic stress

12. The method according to claim 11, wherein said nucleic acid sequence is mRNA or genomic DNA.

13. The method according to claim 11, wherein said examining step is selected from the group consisting of Northern blotting with a suitable nucleic acid probe, reverse-transcriptase PCR, RNase protection analysis and *in situ* hybridization.

14. A method of determining tumorigenic potential of a cell comprising examining said cell for the presence of Chfr polypeptide expression in said cell, wherein the absence of said polypeptide sequence indicates that said cell is predisposed to tumorigenesis upon exposure to mitotic stress.

15. The method according to claim 14, wherein said examining step is selected from the group consisting of Western immunoblotting, enzyme-linked immunoassay, immunofluorescence and immunohistochemistry.

16. A method for determining tumorigenic potential of a cell comprising examining said cell for mutations in the *chfr* gene, wherein the presence of mutations in said gene indicates that the cell is predisposed to tumorigenesis upon exposure to mitotic stress.

17. The method according to claim 16, wherein said examining step comprises performing *in situ* hybridization.

18. The method according to claim 16, wherein said examining step comprises obtaining the nucleic acid sequence of the *chfr* gene in said cell and comparing said sequence to the sequence of a normal *chfr* gene to determine if the *chfr* gene of the cell bears a mutation.

19. The method according to claim 18, wherein said comparing step comprises performing conformation sensitive gel electrophoresis or single strand polymorphism analysis.

20. A method for determining tumorigenic potential of a cell comprising examining said cell for Chfr-mediated ubiquitin-protein ligase activity, wherein the absence of said activity indicates that the cell is predisposed to tumorigenesis upon exposure to mitotic stress.

21. A diagnostic reagent comprising a nucleotide sequence that binds to the *chfr* nucleic acid sequence or a fragment thereof, said reagent sequence associated with a detectable label.

22. The reagent according to claim 21, which is an anti-sense fragment of SEQ ID NO: 1 or a fragment of said SEQ ID NO: 1.

23. A diagnostic reagent comprising a ligand which binds to Chfr, said ligand associated with a detectable label.

24. The reagent according to claim 23 wherein said ligand is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or a recombinant antibody of classes IgG, IgM, IgA, IgD and IgE, a Fab, Fab' or F(ab')₂, or Fc

antibody fragment thereof which binds Chfr, a single chain Fv antibody fragment, a recombinant construct comprising a complementarity determining region of an antibody, a synthetic antibody or a chimeric antibody or a humanized antibody construct which shares sufficient CDRs to retain functionally equivalent binding characteristics of an antibody that binds said Chfr.

25. A diagnostic kit for detecting the tumorigenic potential of a cell, said kit comprising a diagnostic reagent selected from the group consisting of a ligand which binds to Chfr, said ligand associated with a detectable label, and a nucleotide sequence that binds to the *chfr* nucleic acid sequence or a fragment thereof, said reagent sequence associated with a detectable label, and further comprising suitable components for detection of said label.

26. A diagnostic kit for detecting the tumorigenic potential of a cell comprising components for a chfr-mediated ubiquitin protein ligase assay.

27. A composition which inhibits the biological activity of Chfr.

28. The composition according to claim 27, which is a ligand which binds to Chfr and inhibits its biological activity.

29. The composition according to claim 28, wherein said ligand is selected from the group consisting of a polyclonal antibody, a monoclonal antibody or a recombinant antibody of classes IgG, IgM, IgA, IgD and IgE, a Fab, Fab' or F(ab')₂, or Fc antibody fragment thereof which binds Chfr, a single chain Fv antibody fragment, a recombinant construct comprising a complementarity determining region of an antibody, a synthetic antibody or a chimeric antibody or humanized antibody construct which shares sufficient CDRs to retain functionally equivalent binding characteristics of an antibody that binds said Chfr.

30. The composition according to claim 27, which is a chemical compound.
31. A method of identifying a Chfr inhibitor, said method comprising the steps of:
- (a) contacting a cell capable of expressing Chfr with a suitable amount of a test compound, and assessing the level of expression of Chfr in said cell;
 - (b) assessing the level of expression of Chfr in an otherwise identical cell which has not been contacted with said test compound; and
 - (c) comparing the levels of Chfr expression, wherein a lower level of expression of said Chfr in said cell (a) compared with the level of Chfr in said cell (b) indicates that said test compound is a Chfr inhibitor.
32. A Chfr inhibitor identified by the method of claim 31.
33. A method of identifying a Chfr inhibitor, said method comprising the steps of:
- screening a test compound in a Chfr-mediated ubiquitin-protein ligase assay, wherein the substantial absence of, or reduction in, said ligase activity in said assay in the presence of said test compound indicates that said test compound inhibits Chfr function.
34. The method according to claim 33 further comprising the step of contacting a mixture which normally demonstrates Chfr-mediated ubiquitin-protein ligase activity with a test compound; and assaying said mixture and test compound for said activity, wherein the absence of said activity in the presence of said test compound indicates that said test compound inhibits Chfr function.
35. The method according to claim 34, wherein said mixture comprises a labeled Chfr protein, the E1 ligase enzyme, the E2 ligase enzyme, ubiquitin and ATP.

36. The method according to claim 34, wherein said assaying step comprises separating said labeled Chfr protein from said system, and performing gel electrophoresis thereon, and immunoblotting said gel with an anti-ubiquitin antibody, wherein the detection of ubiquitin in the gel by said antibody demonstrates Chfr-mediated ubiquitin-protein ligase activity.

37. The method according to claim 34, wherein said assay is an *in vitro* assay.

38. A Chfr inhibitor identified by the method of claim 34.

39. A method of retarding the growth of a cancer cell, said method comprising administering to said cell a Chfr inhibitor that enhances the sensitivity of said cell to mitotic stress.

40. The method according to claim 39 further comprising administering to said cancer cell an agent which disrupts microtubule function.

41. The method according to claim 39, wherein said method kills said cancer cell.

42. A method of assessing the sensitivity of a tumor cell to an agent which disrupts microtubule function, said method comprising examining said cell for a characteristic selected from the group consisting of:

- (a) the substantial absence of a *chfr* gene;
- (b) the substantial absence of Chfr protein;
- (c) the substantial absence of Chfr-mediated ubiquitin-protein ligase activity; and
- (d) a mutation in the *chfr* gene;

wherein the identification of any of said characteristics provides an indication that said tumor cell is sensitive to an agent which disrupts microtubule function.

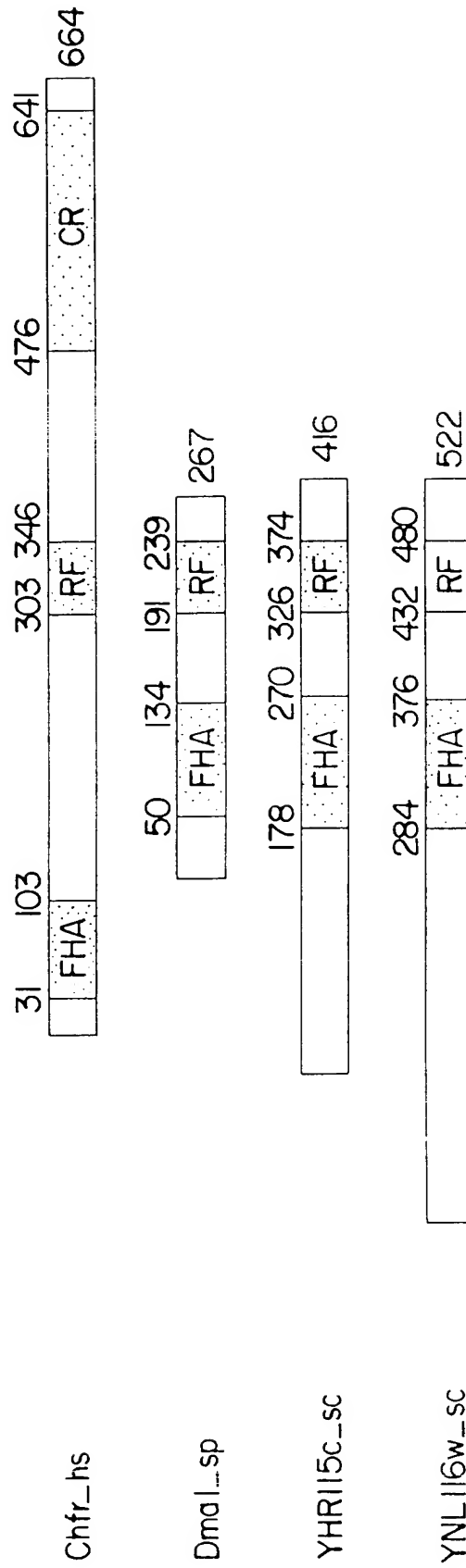


FIG. 1A

[illegible]

Rad53_sc	104	I STNGTWNLGQKVERN....SNQLLSQGDEI TSTSGTVINKLKVVKK....QTCPLQTGDVI
Chfr_hs	77	GSSSGTFLNIHV RLSP PSKTSKPYPISNN DIL
Dra1_sp	104	KSSSGTFLNHQR LSPASSLSKDT PLRDGDIL : : : : : : : : : : : : : : : :
YNL116w_sc	346	S N G T F N R L G D I

FIG. 1C

ICP0_vzv	18	TCT ICMSTVSDI.GKTM..PCDHDFCFV CI RAWTS...TSV QCPLCRCPV	61
Chfr_hs	303	TC I ICQDLLHDCVSLQ..PCMHTFCAACYSGWMERS...SLCPTCRCPV	346
Dmal_ap	191	ECCICLMPVLP CQALFVAPCSHSYHYKCI RPTLN E SHPYFSCF ICRKYH	239
YNL16w_sc	432	DCSICLCK IKP CQAIF I SPCAHSWHFRCVRRRLVMLSYPQFVCPNCRSSC	480
		: :: : : : : : : : : : : : : : : : : : :	
		C IC C HFC C W CP CR	

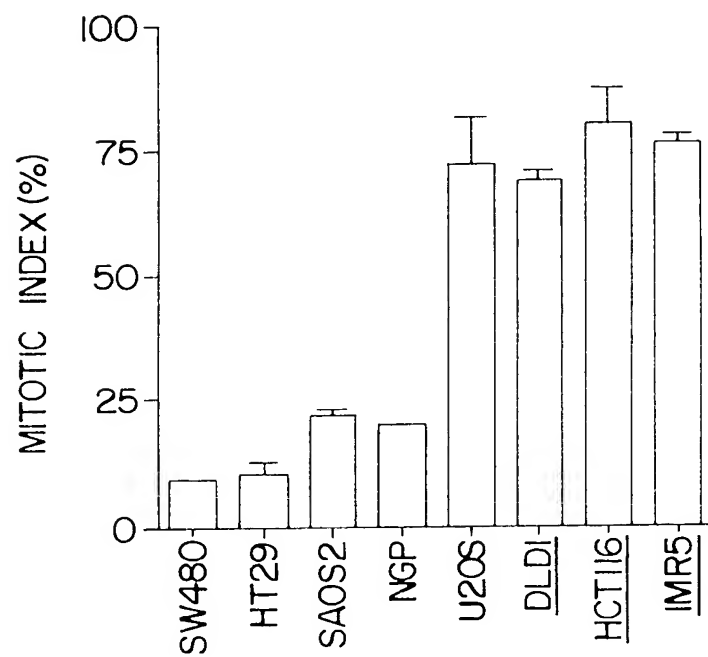


FIG. 2

wt *chfr*

5' ctcGTGgct

3' gagCACcga

L V A

FIG. 3A

U2OS *chfr*

5' ctcATGgct

3' gagTACcga

L M A

FIG. 3B

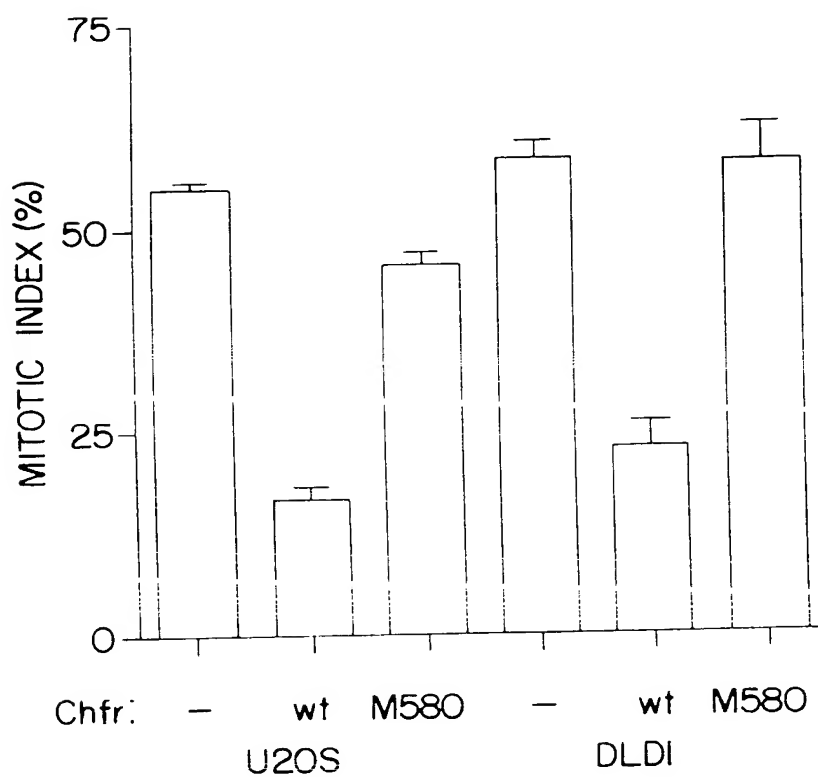


FIG. 3C

FIGURE 4A

aagaattcgg	cacgaggccg	caatgtctct	tgacagcggc	ggcggcgcag	ccggttccgg	60
gttcggcgcg	gggcggggat	gtgaatcccc	atg gag cgg ccc gag gaa ggc aag	114		
			Met Glu Arg Pro Glu Glu Gly Lys			
			1 5			
cag tcg ccg ccg ccg cag ccc tgg gga cgg ctc ctg cgt ctg ggc gcg	162					
Gln Ser Pro Pro Pro Gln Pro Trp Gly Arg Leu Leu Arg Leu Gly Ala						
10 15 20						
gag gag ggc gag ccg cac gtc ctc ctg agg aag cgg gag tgg acc atc	210					
Glu Glu Gly Glu Pro His Val Leu Leu Arg Lys Arg Glu Trp Thr Ile						
25 30 35 40						
ggg cgg aga cga ggt tgc gac ctt tcc ttc ccc agc aat aaa ctg gtc	258					
Gly Arg Arg Arg Gly Cys Asp Leu Ser Phe Pro Ser Asn Lys Leu Val						
45 50 55						
tct gga gat cac tgt aga att gta gtg gat gaa aaa tca ggt cag gtg	306					
Ser Gly Asp His Cys Arg Ile Val Val Asp Glu Lys Ser Gly Gln Val						
60 65 70						
aca ctg gaa gat acc agc acc agt gga aca gtg att aac aag ctg aag	354					
Thr Leu Glu Asp Thr Ser Thr Ser Gly Thr Val Ile Asn Lys Leu Lys						
75 80 85						
gtt gtt aag aag cag aca tgc cct tta cag act ggg gat gtc atc tac	402					
Val Val Lys Lys Gln Thr Cys Pro Leu Gln Thr Gly Asp Val Ile Tyr						
90 95 100						
ttg gtg tac agg aag aat gaa ccg gaa cac aac gtg gca tac ctc tat	450					
Leu Val Tyr Arg Lys Asn Glu Pro Glu His Asn Val Ala Tyr Leu Tyr						
105 110 115 120						
gaa tct tta agt gaa aag caa ggc atg aca caa gaa tcc ttt gaa gct	498					
Glu Ser Leu Ser Glu Lys Gln Gly Met Thr Gln Glu Ser Phe Glu Ala						
125 130 135						
aac aag gaa aat gtg ttc cat ggg acc aaa gat acc tca ggt gca ggt	546					
Asn Lys Glu Asn Val Phe His Gly Thr Lys Asp Thr Ser Gly Ala Gly						
140 145 150						
gca ggg cga ggg gcc gat ccc cgg gtc cct ccg tcg tcg ccc gcc act	594					
Ala Gly Arg Gly Ala Asp Pro Arg Val Pro Pro Ser Ser Pro Ala Thr						
155 160 165						
cag gtg tgc ttt gag gaa cca cag cca cca aca tcg acg tca gac ctc	642					
Gln Val Cys Phe Glu Glu Pro Gln Pro Ser Thr Ser Thr Ser Asp Leu						
170 175 180						
ttc ccc aca gcc tcg gcc tct tcc acg gag cct tct cct gca ggg cga	690					
Phe Pro Thr Ala Ser Ala Ser Ser Thr Glu Pro Ser Pro Ala Gly Arg						
185 190 195 200						
gag cgt tcc tcc agt tgt ggg tct ggg ggt ggt ggc atc tcc cct aaa	738					
Glu Arg Ser Ser Ser Cys Gly Ser Gly Gly Gly Gly Ile Ser Pro Lys						
205 210 215						

FIGURE 4B

gga agt ggt ccc tct gtg gca agt gat gaa gtc tcc agc ttt gcc tca	786
Gly Ser Gly Pro Ser Val Ala Ser Asp Glu Val Ser Ser Phe Ala Ser	
220 225 230	
gct ctc cca gac aga aag act gcg tcc ttt tcg tcg ttg gaa ccc cag	834
Ala Leu Pro Asp Arg Lys Thr Ala Ser Phe Ser Ser Leu Glu Pro Gln	
235 240 245	
gat cag gag gat ttg gag ccc gtg aag aag aaa atg aga gga gat ggg	882
Asp Gln Glu Asp Leu Glu Pro Val Lys Lys Lys Met Arg Gly Asp Gly	
250 255 260	
gac ctt gac ctg aac ggg cag ttg ttg gtc gca caa ccg cgt aga aat	930
Asp Leu Asp Leu Asn Gly Gln Leu Leu Val Ala Gln Pro Arg Arg Asn	
265 270 275 280	
gcc caa acc gtc cac gag gac gtc aga gca gcg gct ggg aag cca gac	978
Ala Gln Thr Val His Glu Asp Val Arg Ala Ala Ala Gly Lys Pro Asp	
285 290 295	
aag atg gag gag acg ctg aca tgc atc atc tgc cag gac ctg ctg cac	1026
Lys Met Glu Glu Thr Leu Thr Cys Ile Ile Cys Gln Asp Leu Leu His	
300 305 310	
gac tgc gtg agt ttg cag ccc tgc atg cac acg ttc tgc gcg gct tgc	1074
Asp Cys Val Ser Leu Gln Pro Cys Met His Thr Phe Cys Ala Ala Cys	
315 320 325	
tac tcg ggc tgg atg gag cgc tcg tcc ctg tgt cct acc tgc cgc tgt	1122
Tyr Ser Gly Trp Met Glu Arg Ser Ser Leu Cys Pro Thr Cys Arg Cys	
330 335 340	
ccc gtg gag cgg atc tgt aaa aac cac atc ctc aac aac ctc gtg gaa	1170
Pro Val Glu Arg Ile Cys Lys Asn His Ile Leu Asn Asn Leu Val Glu	
345 350 355 360	
gca tac ctc atc cag cat cca gac aag agt cgc agt gaa gaa gat gtg	1218
Ala Tyr Leu Ile Gln His Pro Asp Lys Ser Arg Ser Glu Glu Asp Val	
365 370 375	
caa agt atg gat gcc agg aat aaa atc act caa gac atg ctg cag ccc	1266
Gln Ser Met Asp Ala Arg Asn Lys Ile Thr Gln Asp Met Leu Gln Pro	
380 385 390	
aaa gtc agg cgg tct ttt tct gat gaa gaa ggg agt tca gag gac ctg	1314
Lys Val Arg Arg Ser Phe Ser Asp Glu Glu Gly Ser Ser Glu Asp Leu	
395 400 405	
ctg gag ctg tca gac gtt gac agt gag tcc tca gac att agc cag cca	1362
Leu Glu Leu Ser Asp Val Asp Ser Glu Ser Ser Asp Ile Ser Gln Pro	
410 415 420	
tac gtc gtg tgc cgg cag tgt cct gag tac aga agg cag gcg gcg cag	1410
Tyr Val Val Cys Arg Gln Cys Pro Glu Tyr Arg Arg Gln Ala Ala Gln	
425 430 435 440	

FIGURE 4C

cct ccc cac tgc cca gca ccc gag ggc gag cca gga gcc cca cag gcc	1458
Pro Pro His Cys Pro Ala Pro Glu Gly Glu Pro Gly Ala Pro Gln Ala	
445 450 455	
ctg ggg gat gca ccc tcc acg tcc gtc agc ctg acg aca gca gtc cag	1506
Leu Gly Asp Ala Pro Ser Thr Ser Val Ser Leu Thr Thr Ala Val Gln	
460 465 470	
gat tac gtg tgc cct ctg caa gga agc cac gcc ctg tgc acc tgc tgc	1554
Asp Tyr Val Cys Pro Leu Gln Gly Ser His Ala Leu Cys Thr Cys Cys	
475 480 485	
ttc cag ccc atg ccc gac cgg aga gcg gag cgc gag cag gac ccg cgt	1602
Phe Gln Pro Met Pro Asp Arg Arg Ala Glu Arg Glu Gln Asp Pro Arg	
490 495 500	
gtc gcc cct cag cag tgt gcg gtc tgc ctg cag cct ttc tgc cac ctg	1650
Val Ala Pro Gln Gln Cys Ala Val Cys Leu Gln Pro Phe Cys His Leu	
505 510 515 520	
tac tgg ggc tgc acc cgg acc ggc tgc tac ggc tgc ctg gcc ccg ttt	1698
Tyr Trp Gly Cys Thr Arg Thr Gly Cys Tyr Gly Cys Leu Ala Pro Phe	
525 530 535	
tgt gag ctc aac ctg ggt gac aag tgt ctg gac ggc gtg ctg aac aac	1746
Cys Glu Leu Asn Leu Gly Asp Lys Cys Leu Asp Gly Val Leu Asn Asn	
540 545 550	
aac agc tac gag tca gac atc ctg aag aat tac ctg gca acc aga ggt	1794
Asn Ser Tyr Glu Ser Asp Ile Leu Lys Asn Tyr Leu Ala Thr Arg Gly	
555 560 565	
ttg aca tgg aaa aac atg ttg acc gag agc ctc gtg gct ctc cag cgg	1842
Leu Thr Trp Lys Asn Met Leu Thr Glu Ser Leu Val Ala Leu Gln Arg	
570 575 580	
gga gtg ttt ctg ctg tct gat tac aga gtc acg gga gac acc gtt ctg	1890
Gly Val Phe Leu Leu Ser Asp Tyr Arg Val Thr Gly Asp Thr Val Leu	
585 590 595 600	
tgt tac tgc tgt ggc ctg cgc agc ttc cgt gag ctg acc tat cag tat	1938
Cys Tyr Cys Cys Gly Leu Arg Ser Phe Arg Glu Leu Thr Tyr Gln Tyr	
605 610 615	
cgg cag aac att cct gct tcc gag ttg cca gtg gcc gta aca tcc cgt	1986
Arg Gln Asn Ile Pro Ala Ser Glu Leu Pro Val Ala Val Thr Ser Arg	
620 625 630	
cct gac tgc tac tgg ggc cgt aac tgc cgc act cag gtg aaa gct cac	2034
Pro Asp Cys Tyr Trp Gly Arg Asn Cys Arg Thr Gln Val Lys Ala His	
635 640 645	
cac gcc atg aaa ttc aat cat atc tgt gaa caq aca agg ttc aaa aac	2082
His Ala Met Lys Phe Asn His Ile Cys Glu Gln Thr Arg Phe Lys Asn	
650 655 660	

FIGURE 4D

taagcatcca gaggcctga gcagctttca gcactggagg tgaagagagc gtgttttttaa 2142
 aatacagaga caagcacgtc aaggtgtttt cacagccccc tgaggggaagg gacgcagggt 2202
 ctccgacagg tgctctgggg tgactcttct gtggagcttt ttaccctctg agtgagaccc 2262
 tccccagagc cccggggggc gcagcccgcc ctcttggtga gcgctgggca gggctcgtgg 2322
 tggcatcagc agcagagacg aagcctttct gtaacatgcg gccgtcccg cagagaggggc 2382
 agttttgctc ttttgtacat ttccgaaac tacagttaaa gcagaagtct gttttcagga 2442
 aaagtttcaa gggagaaggg caagtattat aaaaacattg ttccaggaga agggagcata 2502
 agttttacagc ctacaggacg tacacaatat cctgctgctg ggaaaaccac agcattttat 2562
 ctatttttta ttttaatagg tttggtgctt atcttctaata aagattttaa tgtcacaac 2622
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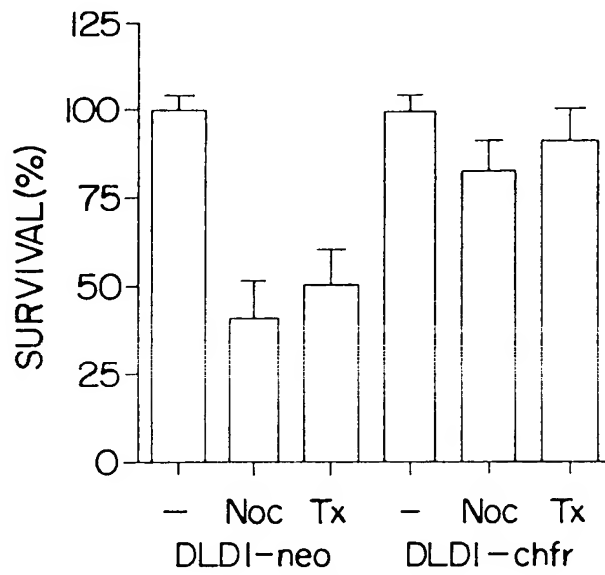


FIG.5

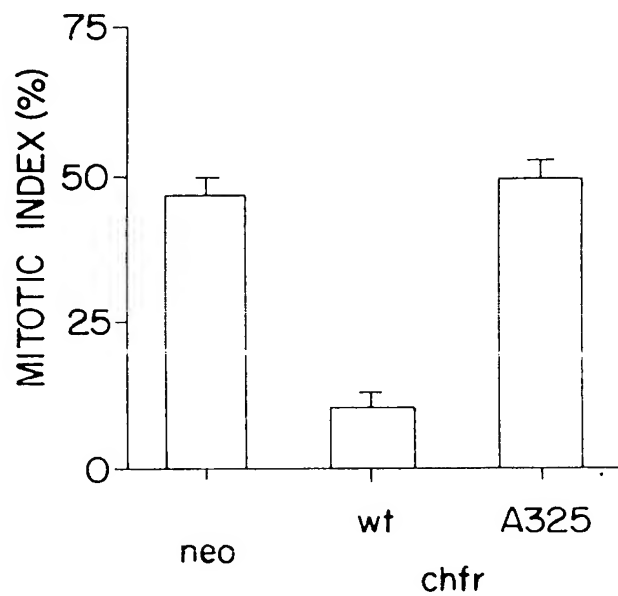


FIG.6

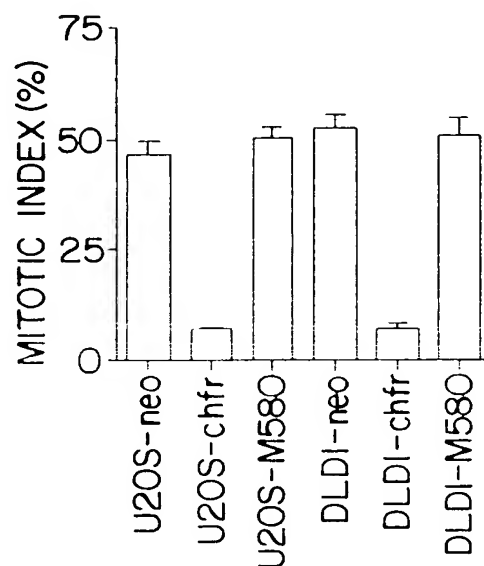


FIG. 7A

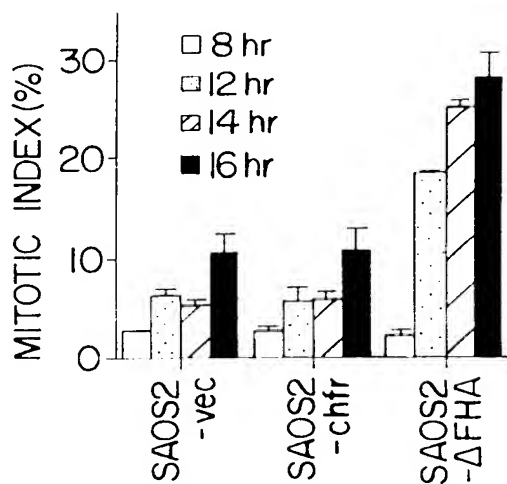


FIG. 7B

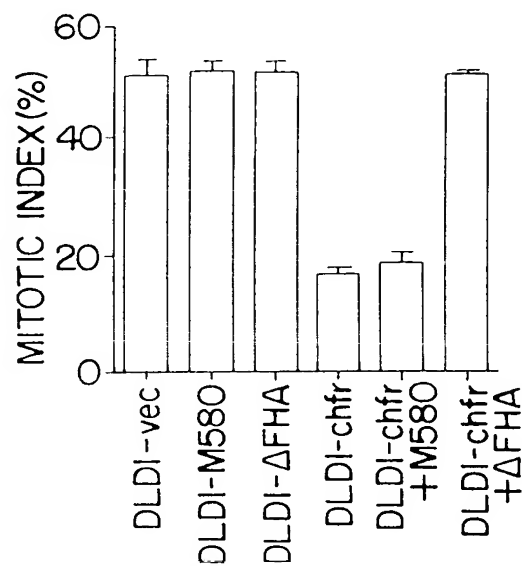


FIG. 7C

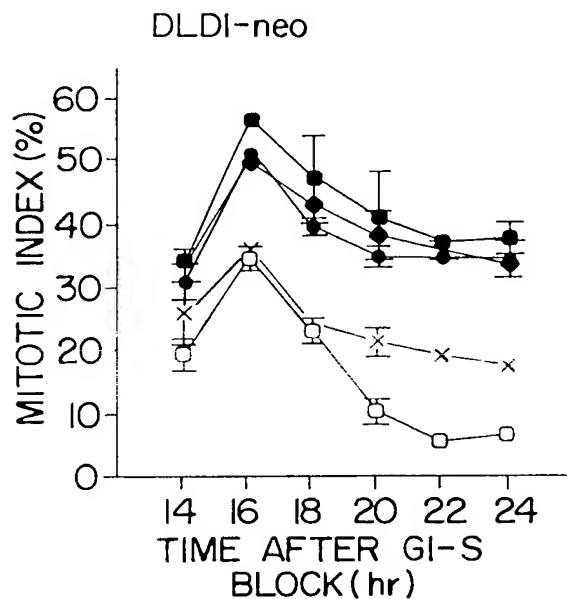


FIG.8A

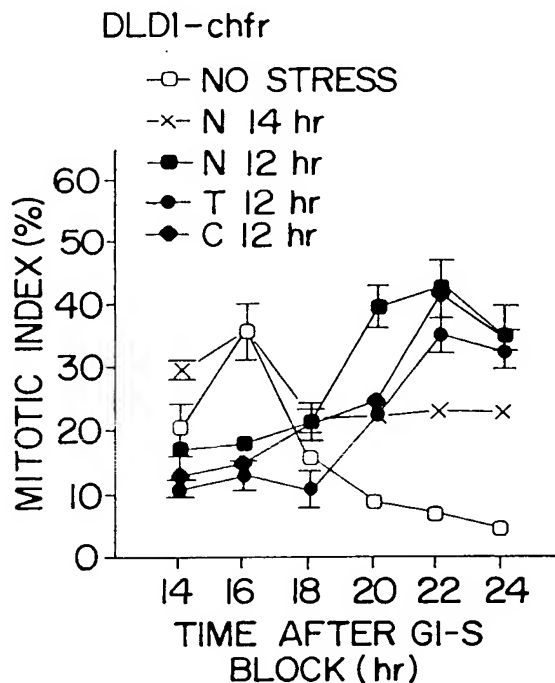


FIG.8B

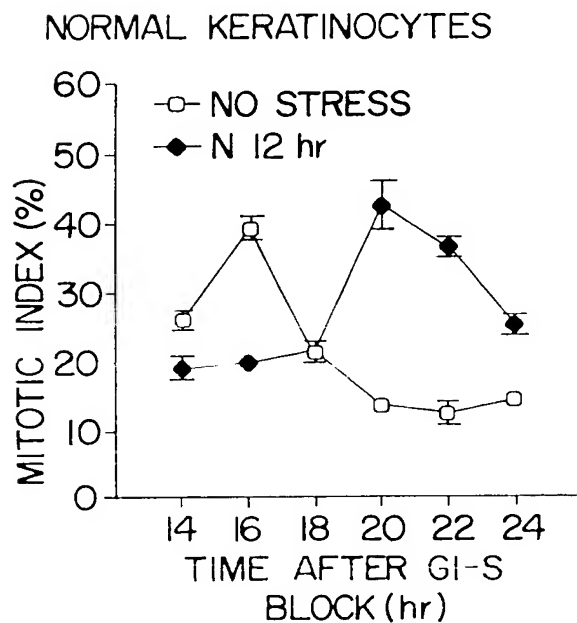


FIG.8C

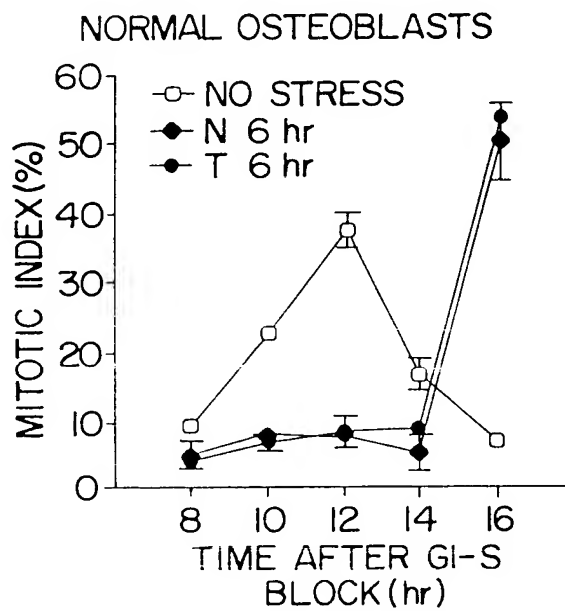


FIG.8D

SEQUENCE LISTING

8813 Rec'd PCT/INTL 24 JAN 2002

<110> The Wistar Institute of Anatomy & Biology
Halazonetis, Thanos
Scolnick, Daniel

<120> Compositions and Methods to Enhance Sensitivity of
Cancer Cells to Mitotic Stress

<130> WST97APCT

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<150> 60/146,194

<151> 1999-07-29

<160> 32

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Met Glu Arg Pro Glu Glu Gly Lys
1 5

cag tcg ccg ccg ccg cag ccc tgg gga cgg ctc ctg cgt ctg ggc gcg 162
Gln Ser Pro Pro Pro Gln Pro Trp Gly Arg Leu Leu Arg Leu Gly Ala
10 15 20

gag gag ggc gag ccg cac gtc ctc ctg agg aag cgg gag tgg acc atc 210
Glu Glu Gly Glu Pro His Val Leu Leu Arg Lys Arg Glu Trp Thr Ile
25 30 35 40

ggg cgg aga cga ggt tgc gac ctt tcc ttc ccc agc aat aaa ctg gtc 258
Gly Arg Arg Arg Gly Cys Asp Leu Ser Phe Pro Ser Asn Lys Leu Val
45 50 55

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/16391

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; C07K 16/00

US CL : 536/23.1; 530/387.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MPSRCH, DIALOG, WEST

search terms: chfr, cancer, forkhead-associated protein, Ring finger

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	SCOLNICK et al. CHFR prevents chromosomal condensation in response to a defective spindle. Proceedings Amer. Assoc. Cancer Res. March 1999, Vol. 40, page 214, abstract No. 1422. See entire document.	1,7 ----- 3,4,11-13
Y	MURONE et al. The fission yeast dmal gene is a component of the spindle assembly checkpoint, required to prevent septum formation and premature exit from mitosis if spindle function is compromised. EMBO J. 02 December 1996, Vol. 15, No. 23, pages 6605-6616. See entire document.	23-25
X,P	Database GenBank, Accession No. AK001658, ISOGAI et al. 'NEDO human cDNA sequencing project'. Publicly available 16 February 2000. See entire document.	23-25

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-5230	Authorized officer MINH TAM DAVIS Telephone No. (703) 306-0916

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For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

WO 01/09150 A3

(54) Title: COMPOSITIONS AND METHODS TO ENHANCE SENSITIVITY OF CANCER CELLS TO MITOTIC STRESS

(57) Abstract: An isolated nucleic acid sequence of a mitotic checkpoint gene, *chfr*, encodes a Chfr protein having a Forkhead-associated domain and a Ring Finger. This protein is required for regulation of the transition of cells from prophase to metaphase during mitosis. The *chfr* nucleic acid and Chfr polypeptide are useful in diagnosing tumorigenic cells and in screening for drugs which can inhibit the activity of Chfr in a cancer cell, thereby rendering the cell more sensitive to additional anti-tumor therapies.